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Selection of Type I and Type II methanotrophic proteobacteria in a fluidized bed reactor under non-sterile conditions

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ABSTRACT

Type II methanotrophs produce polyhydroxybutyrate (PHB), while Type I methanotrophs do not. A laboratory-scale fluidized bed reactor was initially inoculated with a Type II *Methylocystis*-like dominated culture. At elevated levels of dissolved oxygen (DO, 9 mg/L), pH of 6.2–6.5 with nitrate as the N-source, a *Methylobacter*-like Type I methanotroph became dominant within the biofilms which did not produce PHB. A shift to biofilms capable of PHB production was achieved by re-inoculating with Type II *Methylosinus* culture, providing dissolved N_2 as the N-source, and maintaining a low influent DO (2.0 mg/L). The resulting biofilms contained both Types I and II methanotrophs. Batch tests indicated that biofilm samples grown with N_2 became dominated by Type II methanotrophs and produced PHB. Enrichments with nitrate or ammonium were dominated by Type I methanotrophs without PHB production capability. The key selection factors favoring Type II were N_2 as N-source and low DO.

1. Introduction

Biodegradable plastics derived from renewable biomass sources have become an attractive alternative to petroleum-based products in recent years (DiGregorio, 2009). Polyhydroxyalkanoates (PHAs), which can be used as bioplastics (Reddy et al., 2003), are intracellular granules that accumulate in some bacteria in response to nutrient-limiting conditions in the presence of excess carbon (Madison and Huisman, 1999; Anderson and Dawes, 1990). PHAs are potential substitutes for petroleum-based plastics because they are biodegradable and have similar physical properties (Anderson and Dawes, 1990; Lee, 1996; Ojumu et al., 2004). One of the most commonly studied PHAs is polyhydroxybutyrate (PHB), a polyester produced by many bacteria, including methanotrophs (Anderson and Dawes, 1990; Asenjo and Suk, 1986).

Methanotrophic bacteria are ubiquitous in nature (Hanson and Hanson, 1996; Theisen and Murrell, 2005; Whittenbury et al., 1970) and can be found in abundance at oxic–anoxic interfaces, where oxygen concentrations are low and methane concentrations are relatively high (Hanson and Hanson, 1996). Type I methanotrophs are part of the γ-proteobacteria and use the ribulose monophosphate (RuMP) pathway to assimilate carbon. Type II methanotrophs are part of the α-proteobacteria and utilize the serine pathway to assimilate carbon (Graham et al., 1993). Since the 1990s, most research concerning methanotrophic biofilms in bioreactors has focused on the ability of methanotrophs to biodegrade toxic compounds including trichloroethylene, dichloroethylene, and vinyl chloride (Arvin, 1991; Clapp et al., 1999; Fennell et al., 1992; Jewell et al., 1992; Phelps et al., 1990; Strandberg et al., 1989). Several bench scale studies were also conducted to understand the factors that favor different types of methanotrophs for bioremediation. Graham et al. (1993) noted that Type II methanotrophs were able to outcompete Type I methanotrophs in nitrogen-limited conditions and low oxygen concentrations due to their ability to fix molecular nitrogen. Amaral and Knowles (1995) reported that Type II methanotrophs can out-compete Type I methanotrophs at low oxygen concentrations and excess methane concentrations. Recent studies in our laboratory indicate that PHB accumulation only occurs in Type II methanotrophs (Pieja et al., 2011). Selection of Type II methanotrophs therefore appears essential for growth of methanotrophic communities that can produce PHB.

The aerobic biological fluidized bed reactor (FBR) is a high-rate reactor in which microbial biofilms are attached to an inert carrier, such as sand or activated carbon. The carrier is fluidized by upflow of water through the reactor (Hickey et al., 1991; Jewell et al., 1992; Wu et al., 2008). FBRs can potentially generate large quantities of methanotrophic biomass in a relatively small volume. If sloughed biomass retains the capacity for PHB production, it can then be harvested and used for that purpose. The purpose of this

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study was to examine conditions favorable for selection of Type II over Type I methanotrophs at ambient temperatures and under non-sterile operational conditions, We also examined the PHB production potential of the resulting biofilms.

2. Methods

2.1. Medium and source of methanotrophic cultures

W1 medium was used for growth of inocula. It contained 0.8 mM $MgSO_4$, 10 mM $NaNO_3$, 0.14 mM $CaCl_2$, 1.2 mM $NaHCO_3$, 2.35 mM KH_2PO_4, 3.4 mM K_2HPO_4, 20.7 μM Na_2MoO_4, 10 μM FeEDTA, and 1 mL trace element solution. The trace element solution contained (mg per L): $FeSO_4{\cdot}7H_2O$, 500; $ZnSO_4{\cdot}H_2O$, 400; $MnCl_2{\cdot}7H_2O$, 20; $CoCl_2{\cdot}6H_2O$, 50; $NiCl_2{\cdot}6H_2O$, 10; H_3BO_3, 15; EDTA, 250. W1 medium (50 mL) was sterlized by autoclaving at 121 °C for 40 min to prevent culture contamination in long-term incubations carried out in 158-mL serum bottles. Two methanotrophic cultures were used as inocula. For start-up, we used an enrichment derived from sediment at a hot spring (41 °C) in the eastern foothills of the Sierra Nevada Mountains, CA, hereafter referred to as culture WWHS. This culture was incubated at 37 °C in W1 medium containing no Cu and grew as a flocculent suspension. A 16S rRNA gene clone library analysis indicated that the culture was dominated by Type II methanotrophic proteobacteria with 97% similarity to *Methylocystis parvus*. The second culture inoculated was *Methylosinus trichosporium* OB3b (a gift from the Semrau laboratory, University of Michigan, Ann Arbor, MI). Both cultures were grown under non-sterile conditions. Before inoculation, each culture was transferred from the 158-mL serum bottles and grown in a 2-L glass bottle under a 1:1 methane:oxygen (growth substrates) headspace in W1 medium and horizontally incubated on a shaker operated at 150 RPM at 30 °C. The cultures were harvested for FBR inoculation when OD_{670} exceeded 0.4.

2.2. FBR reactor system and start-up

The study was performed with a non-sterile laboratory scale FBR with a total volume of 15.2 L and granular-activated carbon (GAC) as carrier (Fig. 1). The reaction regime consisted of a glass column with two portions and 11 sampling ports located at various heights. Fluidization occurred in the lower portion of the column (ID = 5 cm, H = 220 cm, volume = 6.7 L); settling occurred in the upper portion (ID = 7.62 cm, H = 50 cm, volume = 7.5 L). Two liters (settled volume) of GAC (Calgon type MRX-P, Calgon Carbon Corp., Pittsburgh, PA, USA) with a mesh size of 10 × 30 was added as carriers in the FBR. The GAC was washed with tapwater prior to use, then fluidized by a peristaltic pump. The influent was prepared with Stanford University tap water and saturated with methane or oxygen gas in gas–water contactors to desired levels of dissolved methane (DCH_4) and dissolved oxygen (DO) then mixed with a nutrient solution (Fig. 1). Gases were supplied from gas cylinders (Praxair, Size T, 2500 PSI). The ratio of DCH_4 to DO in the FBR influent was adjusted by changing gas flow rates into the gas contactors and the flow rate of each solution into the FBR. Water containing DCH_4 and DO was pumped by peristaltic pumps to the recirculation loop and mixed with recycled water prior to delivery at the base of the FBR. A metering pump delivered nutrient solution to the recycle loop from a 70-L polypropylene storage tank. Temperature and pH were monitored with a Royce Model 5000 pH/ORP Analyzer system. The reactor was operated at ambient temperature (20–23 °C) under non-sterile conditions. The increase in fluidized bed height was used as an indicator of the growth of biomass in the FBR when upflow velocity remained constant.

The chemical composition of the tap water included (per L): chloride, 3–16 mg (average 9.5 mg); sulfate, 1.6–38.7 mg (average 18.2 mg); TDS, 27–174 mg (average 95 mg); copper, <20–220 μg (90% percentile: 95 μg); lead, <5.6–21 μg (90% percentile: 5.0 μg); alkalinity (as $CaCO_3$), 8–98 mg (average 49 mg); pH: 8.2–8.7 (average 8.5); NH_4^+, 0.19 mg; NO_3^-, 0.025 mg (Stanford University Utilities Services, 2011). The tap water originated from surface water distributed by the San Francisco Public Utilities Commission through the Stanford University tap water distribution system. This water was used directly (without filtration) for the FBR influent. Its composition was tested directly by Stanford University Utilities Services. The nutrient solution was prepared with distilled water and contained: KH_2PO_4, 44 mM; $FeCl_3{\cdot}H_2O$, 1.85 mM; $NiCl_2{\cdot}6H_2O$, 21.04 μM; $CoCl_2{\cdot}6H_2O$, 21.02 μM; $CuCl_2{\cdot}2H_2O$, 32.37 μM; $MnCl_2{\cdot}4H_2O$, 50.55 μM; Na_2MoO_4, 24.28 μM. The pH of the nutrient solution was maintained between 1.0 and 2.5 by HCl addition. When applicable, nitrate-N was provided as the N-source with 93 mM of $NaNO_3$.

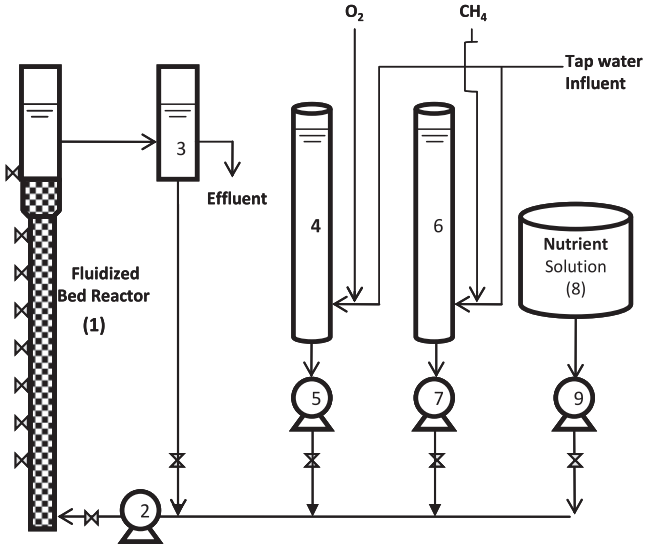

Fig. 1. Schematic diagram of the FBR system for methanotrophic growth. (1) Fluidized bed with sampling points (not all depicted), (2) fluidization pump, (3) effluent recycle reservoir, (4) dissolved O_2 contactor, (5) dissolved O_2 water feed pump, (6) dissolved CH_4 contactor, (7) dissolved CH_4 water feed pump, (8) nutrient solution tank, (9) nutrient solution feed pump.

Prior to inoculation, the FBR was operated for 24-h with recirculation of the influent containing DO (7.2 mg/L) and DCH_4 (4.2 mg/L). On Day 0, WWHS culture (800 mL) was inoculated into the influent port of the FBR, and the reactor was placed in a complete recycle mode, with no influent addition for 2 h to facilitate bacterial adhesion to the GAC. Subsequently, influent containing DO and DCH_4 and nutrient solution was supplied continuously.

The following three operational parameters were evaluated as potential selection pressures: pH, influent DO, and N-source. These parameters were chosen for evaluation based on previous reports of their influence on the growth of Type I and Type II methanotrophs (Amaral and Knowles, 1995; Bowman, 2006; Graham et al., 1993). Influent pH was adjusted by changing the feed rate of the acidified nutrient solution. Influent DO was controlled by adjusting the flow rate of DO-supplemented water and the recirculation rate. When nitrate was the N-source, the nutrient solution contained nitrate. When N_2 was the N-source, the nutrient solution did not contain nitrate; dissolved N_2 in the tap water was the main N-source. Table 1 summarizes the major operational parameters.

2.3. Analytical procedures

2.3.1. Dissolved gas measurements and gas composition analysis

DO concentrations were measured with a portable Hach Sension 6 DO Meter (Hach Chemical, Loveland, CO). To determine

Table 1
Operating parameters and results of the FBR.

Condition	Phase 1	Phase 2.1	Phase 2.2[a]	Phase 2.3	Phase 2.3	Phase 3.1	Phase 3.2	Phase 4
Day of operation	0–15	15–48	48–75	75–107	107–133	133–148	148–229	229–255
pH	6.2–6.5	6.2–6.5	5.5–5.7	6.2–6.5	5.5–5.7	6.2–6.5	6.2–6.5	6.9–7.1
Temperature (°C)	22.8 ± 0.9	21.9 ± 1.1	21.7 ± 1.1	21.9 ± 0.9	22.5 ± 0.7	22.6 ± 0.6	21.7 ± 0.7	21.4 ± 0.7
HRT (min)	8.44	8.44	8.44	8.44	8.44	8.44	8.44	7.60
Primary N-Source	Nitrate	Nitrate	N_2	Nitrate	N_2	N_2	N_2	N_2
Influent DO (mg/L)	4.7 ± 4.5	9.3 ± 0.1	6.9 ± 0.9	7.8 ± 0.9	NC[b]	2.0 ± 0.2	1.1 ± 0.4	1.9 ± 0.5
Influent DCH_4 (mg/L)	9.9 ± 1.3	7.8 ± 2.7	3.2 ± 1.1	4.6 ± 1.4	NC	11.8 ± 1.8	3.5 ± 1.7	4.9 ± 1.4
DO consumption (mg/hr)	143.1 ± 56.0	758.2 ± 31.7	375.0 ± 222.4	296.6 ± 174.8	NC	118.4 ± 15.0	54.4 ± 18.2	115.7 ± 27.9
DCH_4 consumption(mg/hr)	69.9 ± 47.8	285.6 ± 25.9	127.1 ± 60.7	124.0 ± 72.3	NC	80.0 ± 65.7	29.8 ± 20.6	60.7 ± 28.3
O_2:CH_4 use ratio	2.1 ± 1.0	2.7 ± 0.6	2.2 ± 1.0	2.1 ± 1.3	NC	1.5 ± 1.5	2.3 ± 1.5	2.3 ± 2.2
Observed F_e	0.5 ± 0.2	0.7 ± 0.1	0.6 ± 0.3	0.6 ± 0.2	NC	0.3 ± 0.2	0.5 ± 0.2	0.5 ± 0.2
Observed F_s	0.5 ± 0.2	0.3 ± 0.1	0.4 ± 0.3	0.4 ± 0.2	NC	0.7 ± 0.2	0.5 ± 0.2	0.5 ± 0.2
Observed yield (g VSS/g COD)	0.4 ± 0.2	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.1	NC	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Biofilm color	Gray	Pink	Pink	Pink	Pink	Pink	Tan, pink	Tan
Dominant methanotroph	Unknown	Type I[c]	Type I	Type I	Type I	Type I	Mixed[d]	Type II[e]

[a] Nitrate was provided as the N-source until Day 63 and then N_2 was the primary N-source.
[b] Not calculated.
[c] T-RFLP analysis indicated that a strain most closely resembling *Methylobacter* (a Type I methanotroph) dominated.
[d] T-RFLP analysis indicated the presence of *Methylosinus*, *Methylocystis*, and *Methylobacter* spp.-like bacteria.
[e] T-RFLP analysis indicated that Type II methanotrophs (*Methylosinus* and *Methlocystis*) dominated with the presence of *Methylobacter* spp.-like bacteria.

DCH_4 concentrations, samples (25 mL) were withdrawn from the FBR sampling ports and injected into a 58-mL serum bottle that was previously crimp-sealed with a butyl rubber stopper and vacuum-degassed after addition of a 0.2 mL aliquot of 3 N NaOH. The headspace was then equilibrated to ambient pressure by inserting a 23G needle to release the vacuum. The bottle was then shaken to allow methane to equilibrate between the headspace and aqueous phase. Gas phase sample (0.1 mL) was manually injected into a HP 5890 Gas Chromatograph (GC) equipped with Flame Ionization Detector (FID) and Supelco 80/120 Carbopack BDS column (Sigma–Aldrich Corp.) at oven temperature 200 °C with He as carrier gas. The DCH_4 concentrations were then calculated using Henry's Constant, 0.665 atm-m^3/mol (25 °C) (Thibodeaux, 1979). Gas composition (O_2, CH_4, N_2, and CO_2) in the headspace of serum bottles during batch tests was measured using a GOW-Mac GC (GOW-Mac Instrument Co., Bethlehem, PA) equipped with a thermal conductivity detector (TCD) with a CTR1 column (Alltech Associates Inc., Deerfield, IL) with He as carrier gas. The injection volume was 0.3 mL.

2.3.2. Calculation of microbial yield

Observed yield was calculated as the ratio of volatile suspended solids (VSS) produced per gram of substrate (CH_4) consumed as chemical oxygen demand (COD). The fraction of COD assimilated was computed per Rittman and McCarty (2001). For growth with nitrate as the N-source, $Y_{obs} = f_s/(1.98\ g\ COD/g\ VSS)$; for growth with N_2 as the N-source, $Y_{obs} = f_s/(1.63\ g\ COD/g\ VSS)$, where f_s is the fraction of electron equivalents used for cell synthesis ($f_s = 1 - f_e$), and f_e is the fraction of electron equivalents used for energy, f_e = g DO consumed/(g DCH_4 consumed × 4 g COD/g CH_4).

2.3.3. DNA Extraction and pmoA PCR

Biomass samples removed from the FBR were centrifuged and the pellets stored at −80 °C for DNA extraction. Similarly, in batch studies biomass samples were removed from serum bottles, centrifuged, and the pellet stored at −80 °C. DNA was extracted using the FastDNA®SPIN Kit for Soil (Qbiogene) per the manufacturer's protocols. *pmoA* was amplified using the primers 189f and mb661r (Costello and Lindstrom, 1999) labeled with the fluorescent dyes 6-carboxy-fluorescine (A189f) and 5-hexachlorofluorescein (mb661f). Each 30 μL reaction contained 1 × Premix F (Epicentre Biotechnologies), each primer at a concentration of 0.33 μM, and 1 U Failsafe™ Enzyme Mix (Epicentre Biotechnologies). DNA amplification was performed on a GeneAmp® PCR System 9700 (Applied Biosystems) using the following profile: 2 min at 94 °C, 25 cycles of denaturation (1 min at 95 °C), annealing (1.5 min at 55 °C), and extension (1 min at 72 °C); and a final extension of 5 min at 72 °C. In the rare cases where multiple bands were observed, the reaction was re-run at an annealing temperature of 57 °C to eliminate the second band. PCR products were visualized on a 1.5% agarose gel prepared with TAE buffer and stained with ethidium bromide.

2.3.4. Terminal restriction fragment length polymorphism (T-RFLP) analysis

PCR products were purified using Montage® PCR Centrifugal Filter Devices (Milipore) and quantified on a NanoDrop® ND-1000 spectrophotometer. Approximately 30 ng of purified PCR products were digested with a 15 U *Alu*I (New England Biolabs Inc.) in a 1× NE Buffer 4 for 180 min at 37 °C. Digestion products were purified using Montage® PCR Centrifugal Filter Devices (Millipore) and analyzed at MCLAB (South San Francisco, CA). Chromatograms were analyzed using GeneMarker® (SoftGenetics LLC). An *in silico* analysis of known *pmoA* genes was used to identify fragments, and peak identities were confirmed by sequencing *pmoA* from pure cultures.

2.3.5. PHB measurement

Biomass samples were centrifuged and the harvested cells were frozen at −80 °C for at least 2 h prior to freeze-drying. For each sample, approximately 3–6 mg of freeze-dried biomass was added to a 12-mL glass vial with PTFE lined plastic cap (Wheaton Science Products). A slightly modified version of the protocol described by Braunegg et al. (1978) was used. 2 mL of chloroform and 2 mL of acidified methanol (3% (v/v) sulfuric acid) containing 1.0 g/L benzoic acid were added to each vial. The vials were shaken gently and then heated at 100 °C for 3.5 h. Once the samples had cooled to room temperature, 1.0 mL of deionized water was added to each. The vials were subject to vortex mixing for 30 s and allowed to stand until phase separation was complete. The organic phase was analyzed using an Agilent 6890N GC equipped with an HP-5 column (containing (5% phenyl)-methylpolysiloxane, Agent Technologies) and FID. DL-β-hydroxybutyric acid sodium salt (Sigma) was used as a standard.

2.3.6. Batch tests

Batch tests were performed to characterize the FBR biofilm biomass under various conditions. Duplicates were used for all tests. The biomass was withdrawn from the FBR and stored at 4 °C for less than 72-h prior to each test. Cells in the biofilms were suspended by vortex mixing. The concentration of biomass was then determined as volatile suspended solids (VSS) and total suspended solids (TSS) according to *Standard Methods* (APHA, 1985). Bacteria were inoculated into 158-mL serum bottles with 50-mL of W1 medium (in the presence or absence of 10 mM $NaNO_3^-$, as desired) capped with an autoclaved rubber butyl stopper and crimped with an aluminum cap. For biomass grown with nitrate or ammonium, the headspace of the serum bottles was pressurized with He (1.3 atm) after alternatively vacuum-degassing and refilling with He eight times (1 min vacuum-degas and 1 min He refill). Pressure in the headspace was released using a 23G needle to obtain ambient atmospheric pressure prior to further addition of O_2 and CH_4 gases. For biomass grown with N_2 as the sole N-source, the headspace was refilled with N_2 gas instead of He. Each serum bottle was inoculated with 2.5 mL of culture ($OD_{670} \geqslant 0.4$) and pressurized by adding 20 mL O_2 and 30 mL of CH_4 gas. These bottles were then incubated on a shaker at 150 rpm at room temperature (22 °C). Gas samples (0.5 mL) were periodically withdrawn for analysis. Aqueous samples (1.0 mL) were periodically withdrawn from the serum bottle to measure OD_{670} with a Hach DR2800 Spectrometer. When oxygen was exhausted, but methane remained, O_2 gas was added to the headspace. For a test at different initial O_2 partial pressures, different O_2 volumes were provided to achieve the desired partial pressures. For tests of pH, medium pH was adjusted to a target value by addition of a dilute HCl solution.

2.3.7. Biofilm size characterization

The size and physical parameters of the methanotrophic FBR biomass were characterized using the microscopic measurement method reported by Shieh et al. (1981), Araki and Harada (1994), and Fennell et al. (1992). A Nikon SMZ800 microscope was used to examine pieces of GAC with attached biofilm and larger flocs of biomass removed from the reactor. A spherical shape was assumed for biomass estimates.

3. Results and discussion

The tests for the growth of methanotrophic FBR biofilms extended over a 255 day period, and can be divided into four phases (Table 1): Phase 1 (Days 0–15), a start-up period; Phase 2 (Days 15–113), development of Type I-dominated biofilms; Phase 3 (Days 113–229), development of biofilms containing Type I and Type II methanotrophs; and Phase 4 (Days 229–255), accelerated growth of Type II biofilms. During each phase, batch tests were conducted to characterize the biomass and further explore factors that affected the growth of each methanotroph type.

3.1. Phase 1: FBR start-up (Days 0–15)

Operational conditions during this phase were: DCH_4 concentration of 9–10 mg/L, DO concentration of 4–5 mg/L, nitrate as N-source, and a pH of 6.0–6.5 (Table 1). DO consumption was immediately observed after introduction of the WWHS inoculum. By Day 3, DO consumption in the FBR was 270 mg/hr, but no biofilm was visible on the GAC carrier. In an attempt to accelerate biofilm growth, influent DO was increased to 11.0 mg/L. The increased DO inhibited bacterial growth and decreased DO consumption to less than 100 mg/hr within 10 h. Inhibition of methanotroph growth at high levels of DO has been previously reported for dispersed growth cultures fed methane and methanol. Inhibition was attributed to formaldehyde accumulation to inhibitory levels at high oxygen levels (Costa et al., 2001). Accordingly, influent DO was decreased to between 4 and 5 mg/L on Day 4 and maintained at this level until Day 15. Between Days 4 and 15, no visible biofilm developed and consumption of DO remained low (<100 mg/hr). This result suggests that a one-time increase in DO concentrations caused significant inactivation, perhaps due to accumulation of by-products. To restore growth, the reactor was inoculated with an additional 500 mL of WWHS culture on Day 15, and the influent DO concentration was reduced to 4–5 mg/L for 3 days. DO concentration was then incrementally increased.

3.2. Phase 2: Growth and development of Type I methanotroph-dominated biofilms in the FBR (Days 15–113)

3.2.1. Phase 2.1: Initial growth and development of Type I methanotroph-dominated biofilms (Days 15–48)

During Phase 2.1, influent DO was incrementally increased from 4–5 to 9 mg/L to facilitate more rapid growth. Between Days 15 and 33, the GAC bed expanded to fully occupy the fluidization region of the FBR due to growth of biofilms, with bed height increasing from 87 to >220 cm. DO consumption through the fluidized bed increased from less than 200 to 758 ± 32 mg/hr. The observed DCH_4 consumption rate also increased from less than 100 to 286 ± 26 mg/hr (Fig. 3A). Gray colored biofilms coated the GAC carrier within 7 days after the second inoculation. After 2 weeks, the color shifted to a vibrant pink, and biofilm thickness increased to 0.5–2.5 mm (Fig. 2A). T-RFLP analysis conducted on biofilm samples removed on Day 35 indicated that the dominant species was most similar to *Methylobacter* spp. strains, a Type I methanotroph that was not detected in the inoculum. Because the FBR was not operated under sterile conditions, dominance of the *Methylobacter* spp.-like Type I methanotroph was likely due to contamination and the imposition of selection pressures (DO of 9.0 mg/L, pH of 6.2–6.5, nitrate as the N-source) favorable for growth of Type I methanotrophs.

3.2.2. Phase 2.2: Operational factors influencing the growth of Type I methanotrophs (Days 48–75)

In Phase 2.2, we tested the feasibility of shifting the community structure from Type I-dominated biofilms to Type II-dominated biofilms by: (1) decreasing pH to 5.5–5.7, (2) decreasing influent DO to <7.0 mg/L, and (3) eliminating nitrate from the nutrient solution. These actions were based on published research. Pure Type I culture of *Methylobacter* spp. did not grow at pH < 5.5, while some Type II methanotrophs did grow at this pH (Bowman, 2006). Amaral and Knowles (1995) reported that Type II methanotrophs had a competitive advantage when oxygen was limited and methane was present in excess. Removal of nitrate was expected to favor Type II methanotrophs because they possess nitrogenase and can fix molecular nitrogen (Graham et al., 1993), while most Type I methanotrophs lack this capability (Bowman, 2006).

As biomass had grown to the maximum bed height, excess biomass was removed from on Days 48, 53, 61, and 67 to facilitate continued growth. To halt growth of Type I-dominated biofilms, the pH was decreased to 5.5 on Day 48 and, on Day 49 influent DO was decreased from 9–10 to 6–7 mg/L. Bed height remained stable from Days 49 to 54, indicating no biomass growth during that period. From Days 55 to 61, however, the bed height increased by 56 cm, indicating that biofilm growth had resumed. T-RFLP analysis confirmed that the *Methylobacter* spp.-like Type I methanotroph continued to dominate the biofilms. The results suggest that changes in DO and pH only temporarily disrupted growth of Type I biofilms. Changing the nitrogen source from nitrate to N_2 (Days 63–67), however, had a dramatic impact: the increase in bed height stopped, DO consumption decreased from

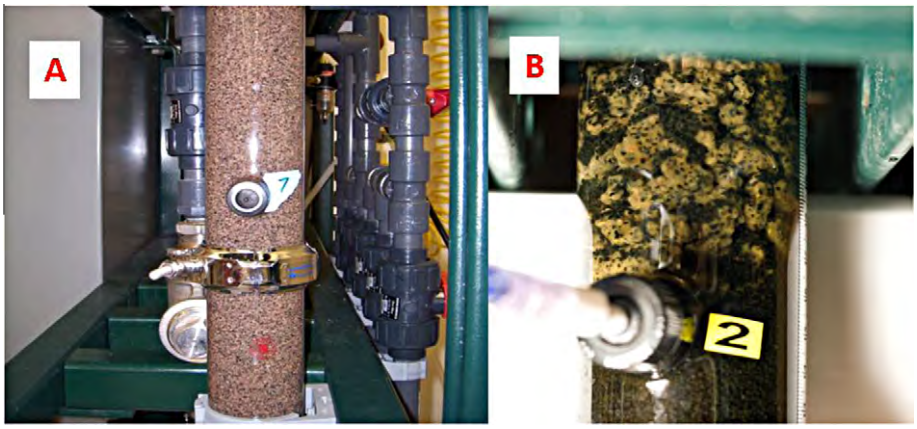

Fig. 2. (A) Left: biomass in the FBR on Day 47. Type I methanotrophic bacteria dominated the biofilm appeared pink in color. (B) Right: biofilms on Day 252 with Type II methanotrophic bacteria as dominant species. The biomass appeared tan in color and formed larger clumps.

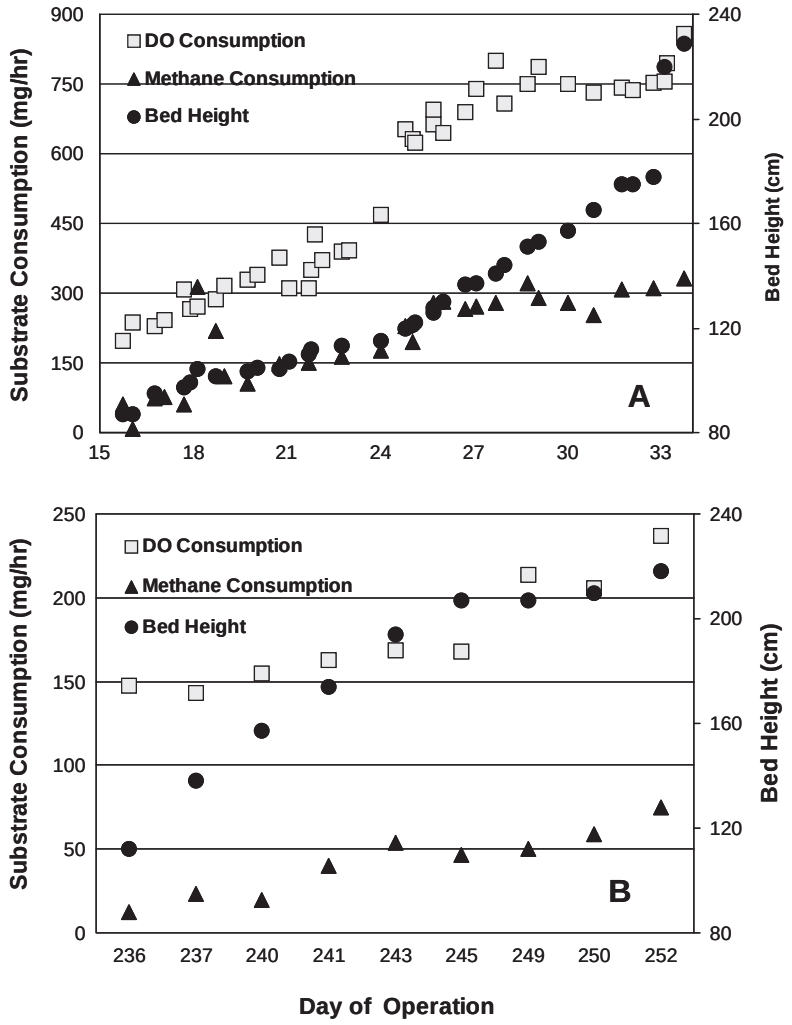

Fig. 3. (A) Increase in bed height, methane consumption, and DO consumption between Days 15 and 33 (Phase 2.1) when Type I-methanotrophic biofilms dominated the FBR. (B) The increase in bed height, methane consumption, and DO consumption between Days 233 and 252 (Phase 4) when Type II-methanotrophic biofilms dominated the FBR. Duplicates were used and the data presented were averaged.

480 to 97 mg/hr, and DCH_4 consumption decreased from 232 to 51 mg/hr, indicating no growth and even death of Type I methanotrophs.

Samples of biomass were removed on Days 62, 69, and 72 for PHB analysis. None of these samples accumulated PHB when incubated under nitrogen-limiting conditions, consistent with prior

research indicating that the Type I methanotrophic cultures do not produce PHB (Pieja et al., 2011).

3.2.3. Confirmation of growth conditions for Type I methanotrophs (Days 75–133)

To further explore conditions that select for Type I-dominated biofilms, the original operational conditions were re-established. On Day 75, the pH was restored to 6.2 and 6.5, influent DO was increased to 8.5–9.0 mg/L, and nitrate was again added. Between Days 78 and 90, pink-colored biofilms began to re-grow, and there was a slow but steady increase in substrate consumption. Over a 12-day span, DO consumption increased from 64 to 172 mg/hr and DCH_4 consumption from 26 to 109 mg/hr. A sharper increase in substrate consumption occurred between Days 90 and 102, and the bed expanded to its maximum height of 220 cm. By Day 102, substrate consumption rates were similar to those measured on Day 48 when *Methylobacter* spp.-like Type I methanotrophs had dominated the reactor. T-RFLP analysis (Day 105) indicated that *Methylobacter* spp.-like methanotrophs dominated the community, with no detectable Type II methanotrophs. No PHB was produced by samples of FBR biomass incubated under nitrogen-limited conditions. The results confirmed that the growth conditions applied (neutral pH, relatively high DO and nitrate as the N-source) favored Type I methanotrophs.

On Day 106, operational conditions were changed to select against Type I methanotrophs. A drop in pH and influent DO temporarily inhibited growth. On Day 113, the pH in the reactor received an additional short-term pH shock, with a pH decrease to 3.5 for 5 h. DO and DCH_4 consumption through the bed dropped to less than 1.0 mg/L, indicating inactivation of Type I methanotrophic activity. FBR operation continued for 20 days with no resumption of methane utilization.

3.2.4. The effect of pH, oxygen and nitrogen source on the growth of Type I methanotroph-dominated biomass

On Day 105, biomass was removed from the FBR for batch studies to assess the effects of different pH levels (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0), partial pressure of oxygen in the headspace (0.15, 0.30, 0.40, 0.50, and 0.60 atm), and nitrogen source (NO_3^- and N_2). The Type I-dominated biofilm biomass did not grow at a pH 3.5, 4.0, and 4.5, but rapidly consumed methane and growth at pH levels between 5.0 and 7.0. Growth was optimal at 0.3 atm of oxygen (approximately 8–9 mg/L of DO at equilibrium condition), but inhibited at higher oxygen partial pressures (>0.4 atm). Type I-dominated biomass grew quickly with nitrate as the N-source, but did not grow with N_2 as the N-source. These results were consistent with the observations of FBR operation between Days 48 and 75 when nitrate was removed from the nutrient solution.

3.2.5. Phase 3: Development of biofilms containing Type I and Type II methanotrophs (Days 133–229)

A series of re-inoculation events were performed with *M. trichosporium* OB3b to facilitate growth of Type II methanotrophic biofilms. Strain OB3b was selected because batch tests demonstrated good growth and PHB production with N_2 as the sole N-source (data not shown). While strain OB3b does not form biofilms in monoculture, it is known to adhere to surfaces in the presence of other bacteria (Speitel and Segar, 1995; Fitch et al., 1996; Clapp et al., 1999). Accordingly, re-inoculations were performed without complete removal of the inactivated Type I-dominated biofilms from the GAC carrier. Cultures of *M. trichosporium* OB3b (2-L with $OD_{670} > 0.4$) were introduced into the FBR on 12 occasions between Days 133 and 186. Because strain OB3b can fix molecular nitrogen (Graham et al., 1993) and is sensitive to high levels of oxygen (Costa et al., 2001), the following operational conditions were maintained: pH 6.2–6.5, influent DO at 2.0 mg/L, excess methane in the influent, and dissolved N_2 as sole N-source.

A steady increase in DO and DCH_4 consumption occurred between the first inoculation (Day 133) and Day 147. On Day 147, influent DO increased from 1.9 to 7.0 mg/L over an 8-h period due to a mechanical malfunction. DO consumption decreased from 157 to 27 mg/hr and DCH_4 consumption decreased from 85 to 14 mg/hr. The effect of this accident suggested that growth of strain OB3b with N_2 as the N-source was inhibited at elevated DO levels, perhaps because of inhibition of nitrogenase activity (Chu and Alvarez-Cohen, 1998; Takeda, 1988). Accordingly, influent DO was decreased to1.5 mg/L or less, and growth gradually resumed between Days 149 and 229. By Day 203 new biofilms had formed on the GAC, and the bed height increased to its maximum.

The physical characteristics of the biofilms began to change soon after the first inoculation with strain OB3b on Day 133. By Day 203, three different regions were discernible. In the lower portion of the bed (150 cm and below), where DCH_4 and DO concentrations were elevated, light tan, almost white, biofilms covered the GAC. In the mid-region (150–220 cm), where DO concentrations were low, pink-colored Type I methanotroph-like biofilms persisted. At the top of the reactor, light tan biofilms attached to the glass wall. This region contained a low level of dissolved oxygen (<0.5 mg/L). T-RFLP analyses indicated that biofilms in each of the three regions contained both Type I metathanotrophs (Methylobacter spp.-like) and Type II methanotrophs (*Methylosinus* and *Methylocystis* spp.-like).

On Day 203, a batch study was performed with biomass samples from the three regions to characterize methane consumption. Nitrate and N_2 gas were evaluated as the N- sources. The results were the same for samples taken from all three regions. When grown with nitrate, the cultures grew to the same pink color as the Type I methanotroph-dominated biomass previously observed in the FBR. T-RFLP analysis confirmed that the resulting cultures were dominated by *Methylobacter* spp.-like species. This suggests that Type I methanotrophs were able to dominate when nitrate was the N-source. The results also suggested that Type I methanotrophic *Methylobacter* spp. may have been persistent due to low level of nitrate in tap water (0.025 mg/L). When N_2 was provided as sole N-source, however, the rate of growth (or methane consumption) was significantly slower than that observed with nitrate, but the biomass retained the same white color as the WWHS inoculum and strain OB3b. T-RFLP analysis indicated that *Methylosinus* and *Methylocystis*-like species dominated these enrichments. When N_2 was provided as the primary N-source, the specific growth rates of biomass samples from the lower portion of the FBR (150 cm and below) were almost 3-fold greater than those of biomass samples removed from the mid-region and top, suggesting a greater abundance of active Type II methanotrophs in the lower portion. At the end of each batch test, the biomass enrichment was incubated under nitrogen-limiting conditions to induce PHB accumulation. No detectable PHB was obtained from samples grown with nitrate (<0.05%, w/w), but PHB levels of 17–26% (w/w) were obtained for all samples grown with N_2.

3.3. Phase 4: Accelerated growth of Type II biofilms (Days 229–255)

In Phase 4, the following measures were implemented to accelerate growth of a Type II-dominated biofilm: (1) removal of biofilms containing Type I methanotrophs in the mid-region of the fluidized bed (150–220 cm); (2) a slight increase in influent DO from <1.5 to 2.0 mg/L; and (3) an increased fluidization rate from 1.8 to 2.0 L/min to more evenly distribute DO. The pH and influent DCH_4 concentration were increased slightly (Table 1). Dissolved N_2 was provided as the N-source, but no nitrate was added in nutrient solution. Over a 19-day period (Day 233–252), bed height

increased from 100 cm to full fluidization (220 cm). DO consumption doubled from 75 to 150 mg/hr and DCH_4 consumption nearly doubled from 39 to 72 mg/hr (Fig. 3.B). The observed yield, Y_{obs} was 0.30 ± 0.12 g VSS/g CH_4 as COD, a value greater than that observed in Phase 2.1 with Type I methanotroph-dominated biofilms (Table 1). The appearance of the new biofilms that dominated the reactor was similar to that of biofilms observed only in the lower portion of the FBR during Phase 3 (Fig 2B). Previous Type I-dominated biofilms formed relatively small granule-like particles. The new biofilms aggregated into large clumps, most noticeably at the bottom of the FBR where methane concentrations were highest.

On Day 254, biomass samples were taken from three different heights in the fluidized bed (31, 61, and 123 cm) for characterization and batch assays. The average diameter of 106 randomly selected biomass particles was 1.9 ± 0.9 mm and ranged between 1.0 and 3.0 mm. Over 20 large particles were removed and examined. The diameter of the particles varied greatly, with the largest exceeding 9.0 mm. After sonication to remove attached biofilm, the average diameter of 149 randomly selected GAC particles was 1.0 ± 0.2 mm. Based on these measurements, the average volume of the biofilm particles, GAC carriers and biofilm biomass was 3.9, 0.50 and 3.40 mm^3, respectively, indicating biomass constituted about 87% of the volume of the particles. The average observed biofilm thickness was 1.9 mm. The VSS and TSS concentrations were highest at the lowest bed height (31 cm) and decreased at higher bed heights (61 and 122.5 cm). Biofilm particles were present at 11.4 g TSS/L and 2.5 gVSS/L at 31 cm and 9.1 g TSS/L with 1.9 g VSS/L at 122.5 cm. Biofilms were thickest and most dense in the lower portion of the FBR where substrate concentrations were highest. By comparison, the range of diameters of 62 randomly selected Type I-dominated biomass samples (Day 35) was slightly less (between 0.5 and 2.0 mm), and more uniform, regardless of location (Fig. 2A).

On Day 254, biomass samples removed from the FBR without any additional growth phase accumulated 6–10% PHB (w/w) after incubation under nitrogen-limiting condition. Samples removed from the middle of the bed (45 cm of bed height) were also taken for enrichment with nitrate, ammonium, or N_2 as N-source. The methane consumption rate in the presence of nitrate or ammonium was rapid with minimal lag, while a significant lag occurred with N_2 as N-source (Fig. 4). T-RFLP results were similar to those obtained in Phase 3: *Methylobacter*-like Type I methanotrophs out-competed Type II methanotrophs when nitrate or ammonium was provided as the N-source. Conversely, when N_2 was the N-source Type II methanotrophs (*Methylosinus* and *Methylocystis*-like) dominated. At the end of the batch tests, biofilm samples enriched with nitrate or ammonium did not contain detectable PHB, while samples grown with N_2 accumulated PHB (~24%, w/w). Almost identical growth patterns were observed with nitrate and ammonium as N-source. This was unlike pure culture observations in which ammonium inhibited growth of methanotrophs, and inhibition was most pronounced with a Type I methanotroph (King and Schnell, 1994). The persistence of Type I *Methylobacter* spp.-like bacteria was likely due to the presence of low levels of nitrate (0.025 mg/L) and ammonium (0.19 mg/L) in the tap water that was used to feed the FBR. If nitrate and ammonium had been eliminated, Type I methanotrophs would likely have been absent.

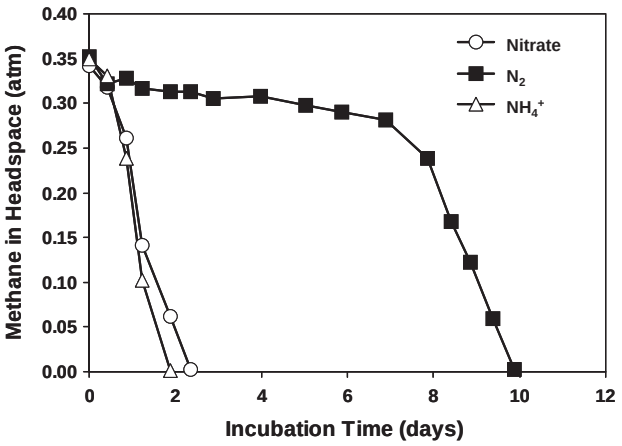

Fig. 4. Batch growth of Type II-dominated biomass grown on different N-sources. Biomass was taken from the FBR on Day 254.

3.4. Implementation of test results

In Phase 2 of this study, *Methylobacter* spp.-like Type I methanotrophs became dominant despite the fact that Type I methanotrophs were not detected in the inoculum. It is possible that *Methylobacter* spp.-like species contaminated the FBR bioflims. After the low pH shock on Day 113, DNA from *Methylobacter* spp.-like methanotrophs was still detected despite inoculation and growth of Type II-biofilms in Phases 3 and 4. When nitrate was provided as the N-source, *Methylobacter* spp.-like bacteria grew in the enrichment indicating that these bacteria can survive with minimal nitrate (and ammonium) supplementation. The *Methlyocystis* added to the FBR in Phase 1 was not detected by T-RFLP analysis during the period of Type I dominance (Days 15–113), but biofilms dominated by both *Methylocystis* and *Methylosinus*-like Type II methanotrophs developed after the operational conditions became favorable for Type II in Phases 3 and 4. This indicates that *Methylocystis* spp.-like methanotrophs can survive under adverse growth conditions and in the presence of Type I-dominated biofilms. The results also demonstrated that under non-sterile condition, both Type I and Type II species can survive and co-exist, and maintenance of a desired methanotroph type requires a specific operational strategy. To grow PHB-producing Type II methanotrophs under non-sterile conditions, use of N_2 as sole N-source and maintenance of a low DO environment proved to be useful selection pressures. In Phase 4, the N-source was present in excess of biomass requirements in the FBR influent. Conditions were therefore, unfavorable for PHB accumulation, and measured PHB levels within the reactor were low (<10%). Higher concentrations up to 20–40% were achievable when harvested biomass was incubated off-line in the absence of nitrogen (i.e., conditions of unbalanced growth).

4. Conclusions

This study demonstrated the feasibility of growing methanotrophic biomass in a FBR under non-sterile conditions and at ambient temperature. It also allowed identification of operational factors of value for selection of Type I and Type II methanotrophs: relatively high DO levels and nitrate addition led to a Type I methanotroph-dominated biofilm; use of N_2 as the N-source and maintenance of relatively low DO concentrations (~2 mg/L) favored growth of Type II methanotrophic biofilms capable of PHB production.

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