


RESEARCH

Open Access



The effect of methane and odd-chain fatty acids on 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) synthesis by a *Methylosinus*-dominated mixed culture

Pawarisa Luangthongkam^{1,2*} , Peter James Strong², Syarifah Nuraqmar Syed Mahamud¹, Paul Evans³, Paul Jensen⁴, Gene Tyson³, Bronwyn Laycock¹, Paul Andrew Lant¹ and Steven Pratt^{1*}

Abstract

A methanotrophic community was enriched in a semi-continuous reactor under non-aseptic conditions with methane and ammonia as carbon and nitrogen source. After a year of operation, *Methylosinus* sp., accounted for 80% relative abundance of the total sequences identified from potential polyhydroxyalkanoates (PHAs) producers, dominated the methane-fed enrichment. Prior to induction of PHA accumulation, cells harvested from the parent reactor contained low level of PHA at 4.0 ± 0.3 wt%. The cells were later incubated in the absence of ammonia with various combinations of methane, propionic acid, and valeric acid to induce biosynthesis of poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). Previous studies reported that methanotrophic utilization of odd-chain fatty acids for the production of PHAs requires reducing power from methane oxidation. However, our findings demonstrated that the PHB-containing methanotrophic enrichment does not require methane availability to generate 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV)—when odd-chain fatty acids are presented. The enrichment yielded up to 14 wt% PHA with various mole fractions of 3HV monomer depending on the availability of methane and odd-fatty acids. Overall, the addition of valeric acid resulted in a higher PHA content and a higher 3HV fraction. The highest 3HV fraction (up to 65 mol%) was obtained from the methane–valeric acid experiment, which is higher than those previously reported for PHA-producing methanotrophic mixed microbial cultures.

Keywords: Methane, Methanotrophs, Polyhydroxyalkanoates, 3-Hydroxybutyrate, 3-Hydroxyvalerate

Introduction

Methane (CH₄) gas, which is a cheap, abundant, and widely available carbon source has emerged as a potential feedstock for the production of polyhydroxyalkanoates (PHAs), a group of biodegradable, biocompatible, and renewable bioplastic (Strong et al. 2016). Many techno-economic studies have demonstrated that the use of methane for PHA production can significantly reduce

raw material cost, minimize environmental impact, and make PHA more attractive in economic perspectives (Rostkowski et al. 2012; Levett et al. 2016; Koller et al. 2017). To date, many methane-utilizing cultures capable of PHA production have been reported in literature (Pfluger et al. 2011; Pieja et al. 2011a, b, 2012; Stein et al. 2011; Zuniga et al. 2011; Belova et al. 2013; Matzen et al. 2013; Rostkowski et al. 2013; Yang et al. 2013; Myung et al. 2015; Sundstrom and Criddle 2015; Criddle and Myung 2016; Myung et al. 2016, 2017). However, in order to make PHA production more competitive also in economic terms, open mixed microbial cultures can be offered as an alternative strategy for producing PHAs at

*Correspondence: uqpluan1@uq.edu.au; risa.luangthongkam@qut.edu.au; s.pratt@uq.edu.au

¹ School of Chemical Engineering, The University of Queensland, St. Lucia, Brisbane, QLD 4072, Australia

Full list of author information is available at the end of the article

lower costs (Broholm et al. 1992; Beun et al. 2002; Salehizadeh and Van Loosdrecht 2004; Dias et al. 2006; Helm et al. 2006, 2008; Albuquerque et al. 2010; Arcos-Hernandez et al. 2010; Colombo et al. 2017; Luangthongkam et al. 2019).

Mixed microbial cultures of methane-utilizing bacteria or methanotrophs, a group of bacteria metabolize methane as their sole carbon and energy source, has a robust and self-regulating nature (Morgan-Sagastume et al. 2010), and offers opportunity to operate under non-aseptic conditions, thereby reducing operating costs of large-scale production (Dias et al. 2006). Under nutrient-limiting conditions (i.e., nitrogen), methanotrophs can convert methane to poly(3-hydroxybutyrate) (PHB), the most common type of PHA naturally produced in microorganisms (Pfluger et al. 2011). PHB is an attractive biopolymer and has comparable properties to conventional polymer (i.e., polypropylene) (Tan et al. 2014); however, it has not made a significant impact in the market because of its narrow melt processing window, high crystallinity, and lack of flexibility (Laycock et al. 2014). One common approach to make PHB with more desirable properties is to incorporate 3-hydroxyvalerate (3HV) into the polymer chain (Laycock et al. 2013), so called poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), which has higher impact strength and flexibility (Sudesh et al. 2000).

The feasibility of PHB and PHBV production in methanotrophic mixed microbial cultures was firstly reported by Myung et al. (2015). The culture dominated by *Methylocystis* sp., one of the most well-known genera of PHA-producing methanotrophs (Pfluger et al. 2011), was shown to produce PHB solely from methane and only produce PHBV when methane was fed with valerate (Myung et al. 2015). Similar approach was further studied in methanotrophic pure cultures of *Methylocystis* and *Methylosinus* sp., but this time with focus on PHA accumulation in both presence and absence of methane (Myung et al. 2016, 2017). In all tested conditions, there is no evidence of either PHB or PHBV synthesis when no methane is available (Myung et al. 2016). Therefore, this led to a conclusion that the methanotrophic utilization of odd-chain fatty acids for PHBV production requires reducing power generated from methane oxidation to produce and/or incorporate 3HV units into the polymer (Myung et al. 2016). However, it should be noted that no study has been done in methanotrophic mixed microbial culture to test its PHA capacity when methane is absent.

Methanotrophic metabolism of PHA has been known to link to the supply of reducing power (Pieja et al. 2011b). It was proposed that PHA functions as a sink of reducing equivalents that can be used to drive cometabolic oxidation (Beccari et al. 1998; Beun et al. 2002;

Dionisi et al. 2005; Çığgın et al. 2007; Ratcliff et al. 2008). For example, PHB-containing methanotrophs showed capacity to transform trichloroethylene (TCE) in the absence of methane (Henry and Grbic-Galic 1991; Henrysson and McCarty 1993; Fitch et al. 1996). Therefore, in this present work, we evaluated PHB and PHBV production in our methanotroph-dominated mixed culture enriched from environmental samples in both presence and absence of methane with combinations of odd-fatty acids (propionic acid and valeric acid). One feature of the work is to consider the potential of stored PHA to support 3HB and 3HV generation, enabling PHBV accumulation in the absence of CH₄.

Materials and methods

Methane-fed cultures

All cultures were grown in modified mineral salt media containing 4 mM MgSO₄·7H₂O, 2 mM KH₂PO₄, 0.4 mM Na₂HPO₄·12H₂O, 0.4 mM CaCl₂·6H₂O, trace elements 0.72 μM FeSO₄·7H₂O, 0.03 μM ZnSO₄·7H₂O, 0.015 μM MnSO₄·7H₂O, 4.8 μM H₃BO₃, 0.2 μM CoCl₂·6H₂O, 5 μM CuCl₂·2H₂O, 0.008 μM NiCl₂·6H₂O, 0.01 μM Na₂MoO₄·2H₂O, 1.7 μM EDTA and vitamins 0.08 μM biotin, 0.045 μM folic acid, 0.15 μM thiamine hydrochloride, 0.22 μM D-pantothenic acid, 0.0007 μM vitamin B12, 0.13 μM riboflavin and 0.4 μM nicotinamide, and amended with 1.6 mL of ammonium stock solution (ammonia solution 28–30%, Merck Millipore, USA) per 1 L of the media.

Inoculum was obtained from sediments from Lake Macdonald and Lake Denwin (Noosa, QLD), and from biomass from the Centre for Solid Waste Bioprocessing (University of Queensland) (Luangthongkam 2019). 6 mg of each sample was inoculated into 160-mL serum bottles (Wheaton, Millville, NJ, USA), with 15 mL medium. The vessels were capped with butyl-rubber stoppers and crimp-sealed. The headspace was filled with air plus 30 mL of a gas mixture (50% CH₄, 50% CO₂). Samples were incubated at room temperature for 4 weeks. Headspace composition was measured periodically. After that, 5 mL of each sample (fully suspended) was taken and combined. This biomass suspension was centrifuged at 10,000g for 5 min (Eppendorf Multipurpose Centrifuge 5804R). Excess fluid was decanted off. The biomass was then inoculated in 250-mL serum bottles (Wheaton, Millville, NJ, USA). The liquid volume was 30 mL, and the headspace volume was 220 mL. The headspace was filled with air plus 50 mL of a gas mixture (50% CH₄, 50% CO₂), added through injection. Once the methane consumption rate was detectable in these samples, an extinction-dilution technique was employed and the mixture was serially diluted to a final dilution of 10⁻⁷. Using the

highest dilution displaying visual turbidity, a 2-L bio-reactor was established using this cell suspension as an inoculum.

Semi-continuous enrichment reactor

A semi-continuous reactor was established using 30 mL of the inoculum suspension described above plus 210 mL of fresh medium with ammonia (13.5 mM) as a nitrogen source. The headspace was filled with air plus 500 mL of the gas mixture (90% CH₄, 10% CO₂). The culture was incubated on a magnetic stirrer, with stirring at 200 rpm at 30 °C. After an incubation period of 15 days, the culture was subjected to a long-term cycling and wasting regime. A repeating cycle was established enabling nearly continuous exponential growth (Fig. 1). In Step 1, 200 mL of fresh medium was added to 40 mL of carry-over culture from the previous cycle, then flushed for 10 min with air. 500 mL of the gas mixture (90% CH₄, 10% CO₂) was then injected into the headspace. In Step 2, the culture was incubated at 30 °C (200 rpm stirring) over a 72-h period. Finally, in Step 3, 200 mL of liquid was quickly removed, completing a cycle. The amount of removed culture determined solids retention time (SRT). The operational SRT was maintained at 3.4 ± 0.3 days SRT over a period of 500 days using a growth repeating cycle (~200 cycles).

PHA accumulation

A portion of the sample removed in Step 3 was centrifuged (6300g) for 5 min (Eppendorf Multipurpose Centrifuge 5804R) and suspended in fresh nitrogen-free W1 medium (Step 4) in 250-mL serum bottles (Wheaton, Millville, NJ, USA) capped with butyl-rubber stoppers and crimp-sealed. The final liquid volume was 30 mL, and the headspace volume was 220 mL. Some samples were amended with 100 ppm of propionic acid (>99% purity, Chem-supply, AUS) and 100 ppm of valeric acid (>99% purity, Sigma-Aldrich, St. Louis, MO, USA), both with and without methane. Tests conditions were categorized as follows: with methane (M1), without methane (M0), propionic acid with methane (M1P), propionic acid without methane (M0P), valeric acid with methane (M1V), and valeric acid without methane (M0V). The bottles were flushed with air before addition of gases as follows: 50 mL of the gas mixture (90% CH₄, 10% CO₂) was added for treatments with methane or 50 mL of nitrogen gas for those without methane. All cultures were grown under non-sterile conditions at 30 °C. After 72 h of incubation, cells were harvested by centrifugation (6300g for 5 min) and dried overnight in an oven at 70 °C. Preserved samples were assayed for PHA content.

Microbial community analysis

Biomass collected from the parent reactor was centrifuged (10,000g) for 5 min (Sigma 1–14 Microcentrifuge)

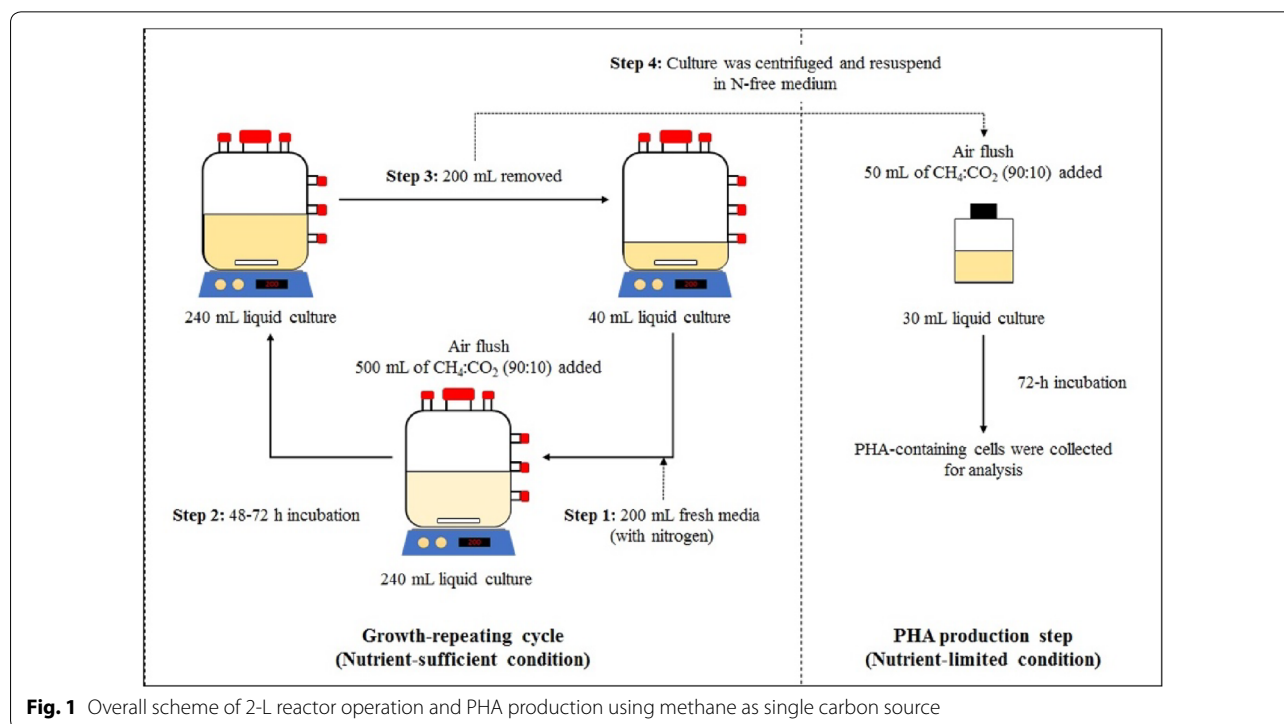


Fig. 1 Overall scheme of 2-L reactor operation and PHA production using methane as single carbon source

and the supernatant discarded. Cell pellets were stored at $-20\text{ }^{\circ}\text{C}$. DNA was extracted from biomass samples using the FastDNA[®] SPIN Kit for Soil (MO BIO Laboratories, Inc.) according to the manufacturer's protocols. Extracted DNA was sent to the Australian Centre for Ecogenomics (ACE) sequencing facility for Illumina iTag sequencing and data processing. Returned 16S rRNA gene sequences that met the quality threshold were clustered using a 95% cutoff and used for community composition analyses. Sequences of closely related species (>97% similarity) were identified by the standard nucleotide BLAST (BLASTn) from NCBI.

PHA analysis

A Perkin-Elmer gas chromatograph was used for PHA monomeric content and composition analysis as previously described by Arcos-Hernandez et al. (2010). The samples were centrifuged at 6300g for 5 min. The supernatant was removed and the pellet dried in an oven at $70\text{ }^{\circ}\text{C}$ overnight. 2 mL of acidified methanol with benzoic acid as internal standard (3% w/w of H_2SO_4) and 2 mL of chloroform were added followed by digestion of the sample for 20 h at $100\text{ }^{\circ}\text{C}$. After cooling to room temperature, 1 mL of MilliQ water was added to allow for phase separation. After 1 h settling, the organic phase was transferred to a vial for GC analysis. Calibration was based on reference standards of a biologically sourced PHBV copolymer (70:30 mol% 3HB: 3HV, Sigma Chemicals). The calibration standards were prepared using the same method. PHA content was reported as a percentage on a dry weight basis (wt%).

Analytical methods

To analyze the concentrations of CH_4 and CO_2 in the headspace, 5 mL from each reactor was withdrawn using a gas-tight syringe and injected into a Shimadzu GC-2014 gas chromatograph equipped with a Valco GC valve (1 mL sample loop), a HAYESEP Q 80/100 packed column (2.4 m length; 1/8" outside diameter, 2 mm inner diameter) and a thermal conductivity detector (TCD). The chromatograph injector, oven, and detector temperatures were set at 75, 45 and $100\text{ }^{\circ}\text{C}$, respectively, and 28 mL min^{-1} of argon at 135.7 kPa was used as a carrier gas.

To analyze total suspended solids (TSS), 2–5 mL of cell suspension was collected and centrifuged at 6300g for 5 min (Eppendorf Multipurpose Centrifuge 5804R). After discarding the supernatant, the cell pellet was dried in an oven at $70\text{ }^{\circ}\text{C}$ overnight and cooled in a desiccator for 24 h before weighing.

Concentrations of propionic acid and valeric acid were assayed using a gas chromatograph (Agilent Technologies, Model 7890A, USA) equipped with a flame

ionization detector (FID) and a polar capillary column (DB-FFAP) on filtered samples (Millex-GP Syringe Filter Unit SLGP033RS). 1.5 mL of cell suspension was collected and centrifuged at 10,000g for 5 min (Sigma 1–14 Microcentrifuge). An internal standard (1000 ppm stock solution) of mixed volatile fatty acids (VFAs) with 1% formic acid was added into all samples.

Results and discussion

Cell growth in the presence of methane and nitrogen

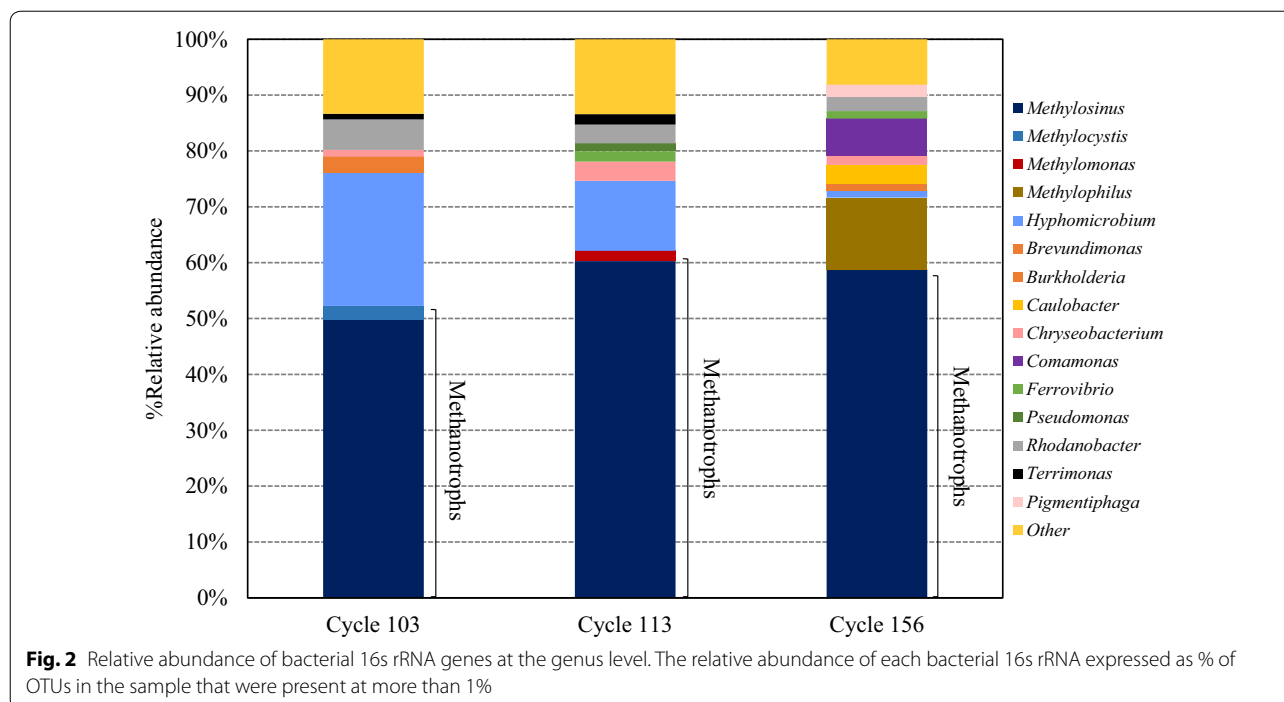
Overall, the culture showed a stable long-term consumption of CH_4 at $1771 \pm 509\text{ }\mu\text{mol of CH}_4\text{ day}^{-1}$. Under these nutrient-sufficient growth conditions, the enrichment showed an exponential growth pattern, doubling every 1.0 days, with the maximum specific growth rate (μ_{max}) being 0.7 day^{-1} . The specific rate of methane oxidation was $18.8 \pm 6.3\text{ mmol CH}_4\text{ g TSS h}^{-1}$, with CO_2 production at $11.4 \pm 4.5\text{ mmol CO}_2\text{ g TSS h}^{-1}$. The biomass concentration was $0.5 \pm 0.1\text{ g TSS L}^{-1}$. Under balanced growth conditions, the active biomass contained $4.0 \pm 0.3\text{ wt\%}$ (with a 3HB: 3HV ratio of 100:0). The accumulation of PHA under such conditions has been reported (Pieja et al. 2011a, b, 2012).

Community flux in the semi-continuous reactor

The community composition at the genus level for the methanotrophic enrichment was monitored by harvesting cells after the 103rd, 113th and 156th growth repeating cycles (or on Days 261, 288, and 393, respectively), as illustrated in Fig. 2. Overall, the members of the community affiliated to the OTU1–25 accounted for 86.7–91.9% of the total sequences identified. All OTUs shared a phylogenetic affiliation at the phylum level, with members of the phyla Proteobacteria and Bacteroidetes accounting for 81.3–90.2% and 1.7–5.3%, respectively (Fig. 2).

Methylosinus was the most abundant genus and accounted for 49.9–58.8% of the total sequences identified, with a non-methane-utilizing methylotroph from genus *Methylophilus* sp. and *Hypomicrobium* being next most abundant (Figs. 2 and 3). *Hypomicrobium* presented at a large proportion initially but was less than 2% relative abundance after the 156th cycle of operation. The population of *Methylophilus* sp. increased from less than 1% relative abundance to 12.9%. Methanotrophic cultures frequently coexist with methylotrophs and heterotrophs that survive on methanotrophic by-products (Ho et al., 2014). In this case, *Methylophilus* and *Hyphomicrobium* are non-methane users who rely on methanol produced as a result of methane oxidation by methanotrophs (Chistoserdova and Lidstrom 2013).

The dominant methanotroph associated with the *Methylosinus* genera shows 97–100% similarity with *Methylosinus trichosporium* OB3b based on best-matched



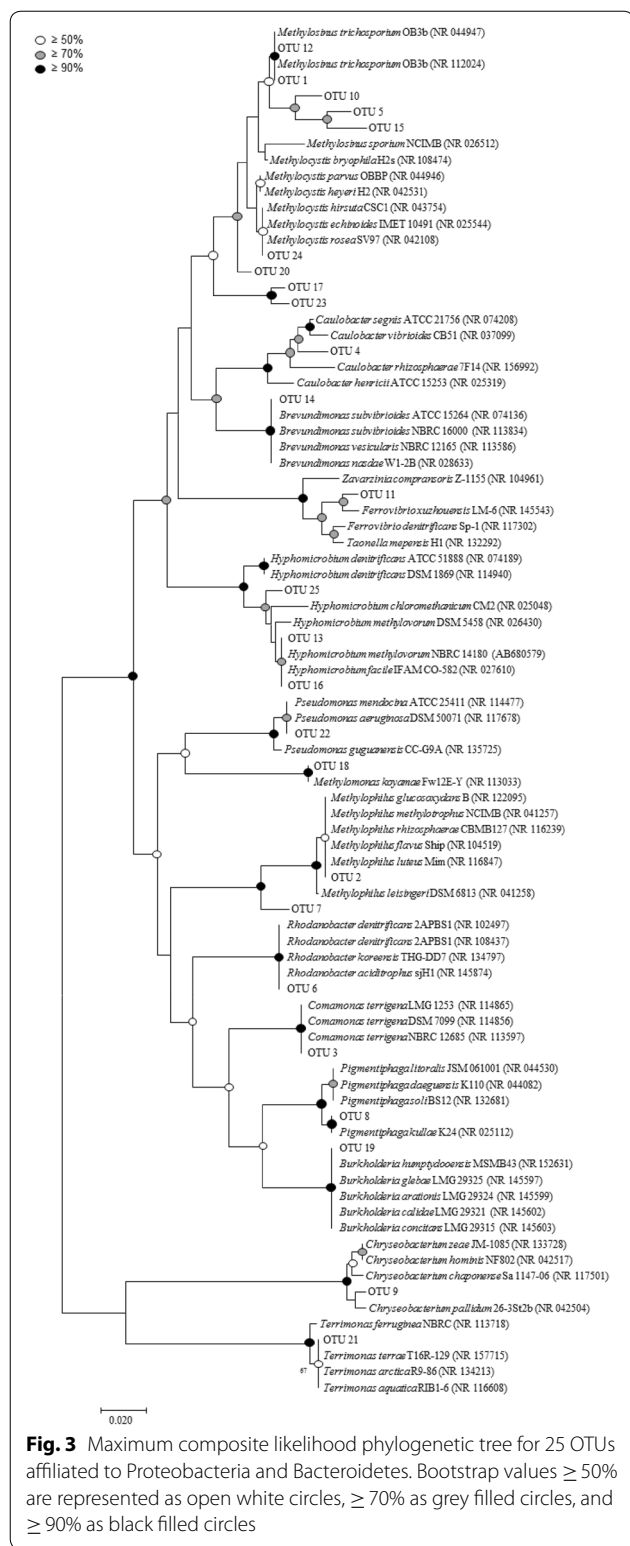
reference sequences using the Basic Local Alignment Search Tool (BLAST) (Additional file 1: Table S1). *Methylosinus trichosporium* OB3b is a well-known PHA-accumulating methanotroph (Garrity et al. 2005), with poly-3-hydroxybutyrate (PHB) constituting from 10 to 50% of the dry weight of cells cultured in nitrogen-free media (Shah et al. 1996; Takeguchi and Okura 2000; Doronina et al. 2008; Xin et al. 2011; Rostkowski et al. 2013; Myung et al. 2016). When its feed is supplemented with valerate, *Methylosinus* is capable of producing poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) under nitrogen-limited conditions (Myung et al. 2016). Myung et al. (2015) reported the highest 3HV fraction at 22.0 ± 3.0 mol% when 100 ppm of valerate was fed with methane (Myung et al. 2016).

Although methane was the sole carbon source in this methane-driven system, heterotrophs—including those capable of accumulating PHA—were observed. For example, *Caulobacter* (OTU4), *Brevundimonas* (OTU14), *Hyphomicrobium* (OTU13, 16, and 25), *Pseudomonas* (OTU22), *Rhodanobacter* (OTU6), *Comamonas* (OTU3), *Burkholderia* (OTU19), and *Terrimonas* (OTU21) (Additional file 1: Table S1) were all present, and these have capacity to accumulate PHA (Urakami and Komagata 1987; Zhao et al. 1993; Khatipov et al. 1998; Silva et al. 2000; Qi and Rehm 2001; Thakor et al. 2003; Keenan et al. 2004; Solaiman and Swingle 2010; Zhu et al. 2010; Li et al. 2011; Tripathi et al. 2012; Wang and Liu 2014). Further, according to previous studies, *Pseudomonas* (Li

et al. 2011; Tripathi et al. 2012), *Comamonas* (Thakor et al. 2003), *Rhodanobacter* (Khatipov et al. 1998) and *Burkholderia* (Silva et al. 2000; Keenan et al. 2004; Zhu et al. 2010) can accumulate copolyesters from propionate or valerate under unbalanced growth conditions. However, the population of these genera presented less than 5% of the total community when the nitrogen starvation experiment was conducted (Additional file 1: Table S1).

Other OTUs in this study, namely, OTU7, 15, and 24, could not be assigned with confidence to known genera at 97% similarity (Additional file 1: Table S1). Thus, a close relative representative sequence was assigned to obtain approximate genus-level taxonomic information. OTU7 was closely related to the genus *Methylophilus* (with 96% similarity); OTU15 was from the genus *Methylosinus* (with 94% similarity); OTU24 showed 96% similarity with *Methylocystis*, a methanotrophic genus known for its capacity to produce PHB and PHBV (Pieja et al. 2011a). *Methylocystis* shows significant similarities to *Methylosinus*, including its stoichiometry of methane oxidation and PHAs formation (Pfluger et al. 2011; Pieja et al. 2011a; Myung et al. 2016).

In short, the community cultivated under methane-driven operation was composed mainly of Proteobacteria with abundance in the range from 84 to 90%. It is well known that almost all methanotrophs belong to this phylum. It should be noted that the population of PHA-producing methanotrophs by Cycle 156th accounted for



80% of total sequences identified from PHA-producing microorganisms.

PHA accumulation

Six conditions were considered, based on combinations of three variables: methane availability, and propionic acid or valeric acid supplementation. The experiments were labeled as follows: with methane (M1), without methane (M0), with methane and propionic acid (M1P), without methane but with propionic acid (M0P), with methane and valeric acid (M1V), and without methane but with valeric acid (M0V). It should be noted that the biomass grown under nutrient-sufficient conditions already contained 4.0 ± 0.3 wt% PHA (with a 3HB: the 3HV ratio of 100:0). Table 1 shows the outcomes from the accumulations. Figure 4 summarizes the final PHA content and fractions of 3HB and 3HV on a dry weight basis (wt%).

In the absence of methane (M0), the PHA content was lowest at 1.3 wt% (with a 3HB:3HV ratio of 100:0) (Fig. 4). The reduction PHA content relative to the initial content is consistent with PHA degradation in the absence of carbon supply, even under unbalanced growth conditions (Beccari et al. 1998; Beun et al. 2002; Dionisi et al. 2005; Çiğgin et al. 2007). The positive control with methane (M1) resulted in a PHA content of 8.6 wt% (with a 3HB:3HV ratio of 100:0) (Fig. 4), which was more than double the initial PHA content and similar to values reported previously (López et al. 2014; Karthikeyan et al. 2015; Zhang et al. 2017). The rates of methane consumption and CO_2 production were 7.1 ± 1.2 mmol CH_4 g TSS⁻¹ h⁻¹ and 2.4 ± 0.3 mmol CO_2 g TSS⁻¹ h⁻¹.

In the presence of propionate (M0P and M1P) there was net PHA consumption, with the final PHA content being below the initial 4 wt%. Still, PHA was produced in both trials, evidenced by the generation of 3HV. In the absence of methane (M0P), the final PHA content was 2.8 wt% (with a 3HB:3HV ratio of 80:20), while in the presence of methane (M1P), the final PHA content was 3.5 wt% (with a 3HB:3HV ratio of 77:23).

The addition of valerate resulted in the highest PHA (and 3HV) production, with the PHA content in the M0V and M1V experiments more than doubling and tripling, respectively. The highest PHA content overall was obtained in the methane and valeric acid (M1V) experiment: 14.1 wt% (with a 3HB:3HV ratio of 35:65). In comparison with previous studies, our *Methylosinus*-dominated enrichment generated very high 3HV content (up to 65 mol%); Myung et al. (2015, 2016) reported 22 mol%, when the same amount of odd-chain fatty acid was added (Myung et al. 2015, 2016).

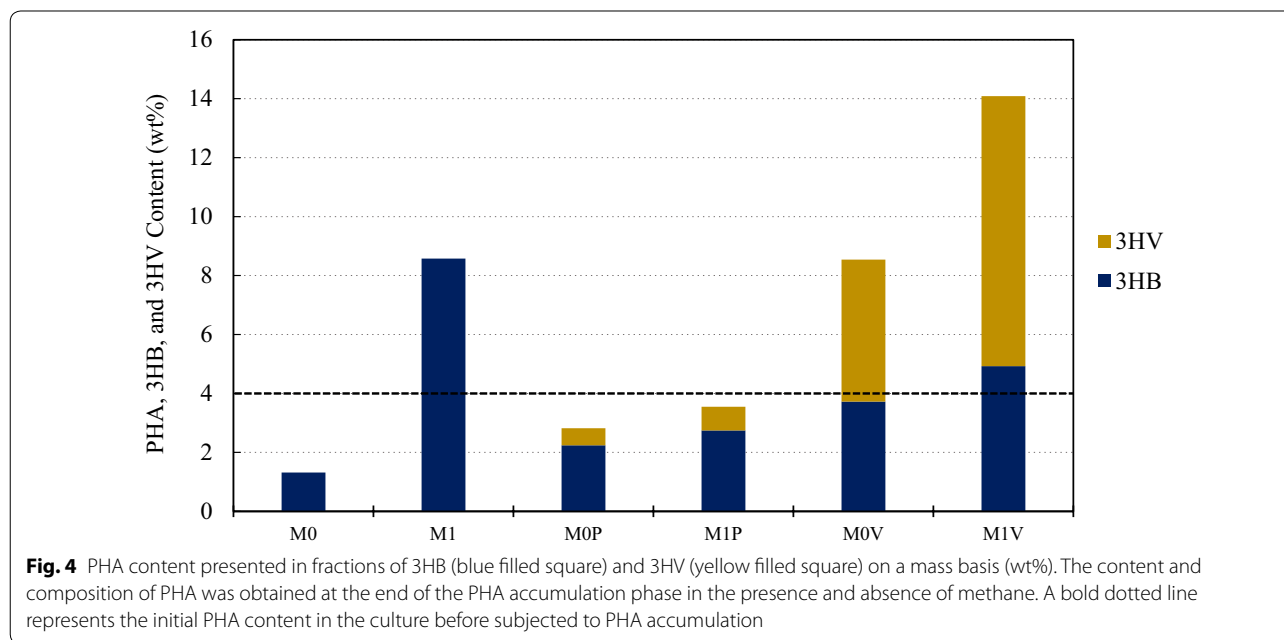
The production of 3HV was unsurprising given that propionic and valeric acids are precursors for propionyl-CoA and valeryl-CoA, respectively, which are intermediates for 3-hydroxyvaleryl-CoA (3HV-CoA) generation (Slater et al. 1998; Myung et al. 2016).

Table 1 PHA content and composition produced by the *Methylosinus*-dominated culture

Odd-chain fatty acids	Methane availability	Code	PHA content (wt%)	3HV fraction (mol%)	Total suspended solid (TSS) (g TSS L ⁻¹)
None	Yes	M1	8.6	0.0	0.42 ± 0.01
	No	M0	1.3	0.0	0.35 ± 0.01
Propionic acid	Yes	M1P	3.5	22.6	0.37 ± 0.01
	No	M0P	2.8	20.5	0.36 ± 0.00
Valeric acid	Yes	M1V	14.1	65.0	0.47 ± 0.00
	No	M0V	8.5	56.4	0.45 ± 0.01

Odd-chain fatty acids	Methane availability	Code	CH ₄ consumed (mmol CH ₄ g TSS ⁻¹ day ⁻¹)	CO ₂ produced (mmol CO ₂ g TSS ⁻¹ day ⁻¹)	C ₃ consumed (mmol C ₃ g TSS ⁻¹)	C ₅ consumed (mmol C ₅ g TSS ⁻¹)
None	Yes	M1	7.1 ± 1.2	2.4 ± 0.3	–	–
	No	M0	–	N/A	–	–
Propionic acid	Yes	M1P	5.2 ± 0.3	1.6 ± 0.3	2.1	–
	No	M0P	–	N/A	1.6	–
Valeric acid	Yes	M1V	4.3 ± 0.4	0.8 ± 0.2	–	2.2
	No	M0V	–	N/A	–	2.3

The content and composition of PHA was measured after 72 h of incubation in the presence and absence of methane with supplementation of odd-chain fatty acids
N/A not applicable



Higher net production in the presence of valeric acid was also unsurprising when compared to the presence of propionic acid. Despite 3HV generation from propionic and valeric acid sharing a common pathway in methanotrophic and heterotrophic microorganisms, a mole of acetyl-CoA, the key precursor for 3HB monomer formation, is required to combine with a mole of propionyl-CoA to form 3HV-CoA, leading to

a competition for acetyl-CoA for 3HB and 3HV formation (Doi et al. 1987; Myung et al. 2016, 2017). Hence, lower formation of 3HV-CoA, as well as net production of PHA, from propionic acid compared to valeric acid, which is a direct precursor to 3HV-CoA, is commonly observed (Berezina 2012; Hao et al. 2016; Myung et al. 2016).

The significant outcome of this work is the generation of PHA in the absence of methane, but with either propionic or valeric acid present. In methanotrophic cultures, the synthesis of PHA is dependent upon the energy available for substrate uptake and assimilation (Pieja et al. 2011b; Myung et al. 2016). The energy is evidently produced via oxidative phosphorylation, which is in turn driven by methane oxidation (Myung et al. 2016), explaining no PHA production when methane is removed during the accumulation phase (Pieja et al. 2011b; Myung et al. 2015, 2016). On the other hand, in this study, although net PHA production was only observed when valeric acid was used, our result shows that PHA synthesis in the absence of methane may have been driven by the reducing power generated via degradation of stored PHA for the assimilation of organic acids. The lower yields for M0P and M0V compared to M1P and M1V are consistent with this hypothesis. Further studies on this topic are required to quantify the relationship between PHA and energetics of odd-chain fatty acids assimilation and incorporation in methanotrophs.

Conclusion

We conclude that a semi-continuous reactor can facilitate methanotrophic enrichment and yield a community capable of producing PHA. The community was dominated by the Proteobacteria with *Methylosinus* being the most abundant genera making up 60% relative abundance. The population accounted for 80% relative abundance of total sequences identified from PHA-producing microorganisms, playing the main role for PHA production. Both propionic acid and valeric acid are able to induce the production of 3HV in a culture that produces pure 3HB on methane alone. A higher fraction of 3HV and PHA content was obtained from the valeric acid treatment. Contrary to previous findings, we did find a synthesis of 3HV in the absence of methane when PHA-stored cells were incubated with either propionic acid or valeric acid. The 3HV fraction reported in this study far exceeds the previously reported values.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s40643-019-0285-1>.

Additional file 1: Table S1. Phylogenetic classification of the 16S rRNA gene sequences (relative abundance > 1%) in methanotrophic-heterotrophic communities.

Abbreviations

PHA: polyhydroxyalkanoate; PHB: poly(3-hydroxybutyrate); PHBV: poly(3-hydroxybutyrate-co-3-hydroxyvalerate); 3HB: 3-hydroxybutyrate; 3HV:

3-hydroxyvalerate; %3HB: 3HB fraction in total PHA (mol 3HB mol PHA⁻¹); %3HV: 3HV fraction in total PHA (mol 3HV mol PHA⁻¹); %PHA: PHA intracellular content (g PHA g TSS⁻¹); SRT: solid retention time; TSS: total suspended solid; VFAs: volatile fatty acids; OTU: operational taxonomic unit.

Acknowledgements

All authors would like to acknowledge funding from the Australian Research Commission (ARC) Discovery Project DP150103060. The Australian Water Management Centre (AWMC) analytical lab for volatile fatty acids, polyhydroxyalkanoates, and alcohols measurements.

Authors' contributions

PAL and PJS carried out the experiments. SNSM performed sample collection and selection. PAL wrote the manuscript with supports from BL. SP and all authors edited the manuscript and give their consent to the final version. SP, PJS, BL, and PAL supervised the project. SP, BL, GT, PJ, and PAL conceived the original idea. All authors read and approved the final manuscript.

Funding

This research was funded by the Australian Research Commission (ARC) Discovery Project DP150103060. Paul Evans is supported by an Australian Research Council Discovery Early Career Research Award (DE170100428).

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional information files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the contributing authors have seen and approved the submission to *Bioresources and Bioprocessing* as an original work.

Competing interests

The authors declare that they have no competing interests.

Author details

¹ School of Chemical Engineering, The University of Queensland, St. Lucia, Brisbane, QLD 4072, Australia. ² Queensland University of Technology, 2 George Street, Brisbane, QLD 4000, Australia. ³ The Australian Centre for Ecogenomics, The University of Queensland, Brisbane, QLD 4072, Australia. ⁴ Advanced Water Management Centre, The University of Queensland, Brisbane, QLD 4072, Australia.

Received: 14 October 2019 Accepted: 29 November 2019

Published online: 17 December 2019

References

- Albuquerque MGE, Torres CAV, Reis MAM (2010) Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses: effect of the influent substrate concentration on culture selection. *Water Res* 44(11):3419–3433. <https://doi.org/10.1016/j.watres.2010.03.021>
- Arcos-Hernandez MV, Gurieff N, Pratt S, Magnusson P, Werker A, Vargas A et al (2010) Rapid quantification of intracellular PHA using infrared spectroscopy: an application in mixed cultures. *J Biotechnol* 150(3):372–379. <https://doi.org/10.1016/j.jbiotec.2010.09.939>
- Beccari M, Majone M, Massanisso P, Ramadori R (1998) A bulking sludge with high storage response selected under intermittent feeding. *Water Res* 32(11):3403–3413. [https://doi.org/10.1016/S0043-1354\(98\)00100-6](https://doi.org/10.1016/S0043-1354(98)00100-6)
- Belova SE, Kulichevskaya IS, Bodelier PL, Dedysh SN (2013) *Methylocystis bryophila* sp. nov., a facultatively methanotrophic bacterium from acidic Sphagnum peat, and emended description of the genus *Methylocystis* (ex Whittenbury et al. 1970) Bowman et al. 1993. *Int J Syst Evol Microbiol* 63(Pt 3):1096–1104. <https://doi.org/10.1099/ijs.0.043505-0>

- Berezina N (2012) Enhancing the 3-hydroxyvalerate component in bioplastic PHBV production by *Cupriavidus necator*. *Biotechnol J* 7(2):304–309. <https://doi.org/10.1002/biot.201100191>
- Beun J, Dircks K, Van Loosdrecht M, Heijnen J (2002) Poly-beta-hydroxybutyrate metabolism in dynamically fed mixed microbial cultures. *Water Res* 36(5):1167–1180
- Brohholm K, Christensen TH, Jensen BK (1992) Modelling TCE degradation by a mixed culture of methane-oxidizing bacteria. *Water Res* 26(9):1177–1185. [https://doi.org/10.1016/0043-1354\(92\)90178-7](https://doi.org/10.1016/0043-1354(92)90178-7)
- Chistoserdova L, Lidstrom ME (2013) Aerobic methylotrophic prokaryotes. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (eds) *The prokaryotes: prokaryotic physiology and biochemistry*. Springer, Berlin, pp 267–285
- Çiğgin AS, Karahan Ö, Orhon D (2007) Effect of feeding pattern on biochemical storage by activated sludge under anoxic conditions. *Water Res* 41(4):924–934. <https://doi.org/10.1016/j.watres.2006.11.017>
- Colombo B, Favini F, Scaglia B, Sciarria TP, D'Imporzano G, Pognani M et al (2017) Enhanced polyhydroxyalkanoate (PHA) production from the organic fraction of municipal solid waste by using mixed microbial culture. *Biotechnol Biofuels* 10(1):201. <https://doi.org/10.1186/s13068-017-0888-8>
- Criddle CS, Myung J (2016) Production of tailored PHA copolymers with methane and added co-substrates. United States patent application 14/825473
- Dias JM, Lemos PC, Serafim LS, Oliveira C, Eiroa M, Albuquerque MG et al (2006) Recent advances in polyhydroxyalkanoate production by mixed aerobic cultures: from the substrate to the final product. *Macromol Biosci* 6(11):885–906. <https://doi.org/10.1002/mabi.200600112>
- Dionisi D, Beccari M, Di Gregorio S, Majone M, Papini MP, Vallini G (2005) Storage of biodegradable polymers by an enriched microbial community in a sequencing batch reactor operated at high organic load rate. *J Chem Technol Biotechnol* 80(11):1306–1318. <https://doi.org/10.1002/jctb.1331>
- Doi Y, Kunioka M, Nakamura Y, Soga K (1987) Biosynthesis of copolyesters in *Alcaligenes eutrophus* H16 from carbon-13 labeled acetate and propionate. *Macromolecules* 20(12):2988–2991. <https://doi.org/10.1021/ma00178a006>
- Doronina NV, Ezhov VA, Trotsenko YA (2008) Growth of *Methylosinus trichosporium* OB3b on methane and poly-β-hydroxybutyrate biosynthesis. *Appl Biochem Microbiol* 44(2):182–185. <https://doi.org/10.1134/S0003683808020099>
- Fitch MW, Speitel GE Jr, Georgiou G (1996) Degradation of trichloroethylene by methanol-grown cultures of *Methylosinus trichosporium* OB3b PP358. *Appl Environ Microbiol* 62(3):1124
- Garrity GM, Bell JA, Lilburn T (2005) Class I. Alphaproteobacteria class. nov. In: Brenner DJ, Krieg NR, Staley JT (eds) *Bergey's manual of systematic bacteriology: volume two the proteobacteria part C the alpha-, beta-, delta-, and epsilonproteobacteria, vol two*. Springer US, Boston, pp 1–574
- Hao J, Wang X, Wang H (2016) Investigation of polyhydroxyalkanoates (PHAs) biosynthesis from mixed culture enriched by valerate-dominant hydrolysate. *Front Environ Sci Eng* 11(1):5. <https://doi.org/10.1007/s11783-017-0896-8>
- Helm J, Wendlandt KD, Rogge G, Kappelmeyer U (2006) Characterizing a stable methane-utilizing mixed culture used in the synthesis of a high-quality biopolymer in an open system. *J Appl Microbiol* 101(2):387–395. <https://doi.org/10.1111/j.1365-2672.2006.02960.x>
- Helm J, Wendlandt KD, Jechorek M, Stottmeister U (2008) Potassium deficiency results in accumulation of ultra-high molecular weight poly-beta-hydroxybutyrate in a methane-utilizing mixed culture. *J Appl Microbiol* 105(4):1054–1061. <https://doi.org/10.1111/j.1365-2672.2008.03831.x>
- Henry SM, Grbic-Galic D (1991) Influence of endogenous and exogenous electron donors and trichloroethylene oxidation toxicity on trichloroethylene oxidation by methanotrophic cultures from a groundwater aquifer. *Appl Environ Microbiol* 57(1):236
- Henrysson T, McCarty PL (1993) Influence of the endogenous storage lipid poly-beta-hydroxybutyrate on the reducing power availability during cometabolism of trichloroethylene and naphthalene by resting methanotrophic mixed cultures. *Appl Environ Microbiol* 5:1602–1606
- Ho A, de Roy K, Thas O, De Neve J, Hoefman S, Vandamme P et al (2014) The more, the merrier: heterotroph richness stimulates methanotrophic activity. *Int Soc Microbiol Ecol* 8(9):1945–1948. <https://doi.org/10.1038/ismej.2014.74>
- Karthikeyan OP, Chidambarampadmavathy K, Nadarajan S, Lee PK, Heimann K (2015) Effect of CH₄/O₂ ratio on fatty acid profile and polyhydroxybutyrate content in a heterotrophic-methanotrophic consortium. *Chemosphere* 141:235–242. <https://doi.org/10.1016/j.chemosphere.2015.07.054>
- Keenan TM, Tanenbaum SW, Stipanovic AJ, Nakas JP (2004) Production and characterization of poly-β-hydroxyalkanoate copolymers from *Burkholderia cepacia* utilizing xylose and levulinic acid. *Biotechnol Prog* 20(6):1697–1704. <https://doi.org/10.1021/bp049873d>
- Khatipov E, Miyake M, Miyake J, Asada Y (1998) Accumulation of poly-β-hydroxybutyrate by *Rhodobacter sphaeroides* on various carbon and nitrogen substrates. *FEMS Microbiol Lett* 162(1):39–45. <https://doi.org/10.1111/j.1574-6968.1998.tb12976.x>
- Koller M, Marsalek L, de Sousa Dias MM, Braunegg G (2017) Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. *N Biotechnol* 37:24–38. <https://doi.org/10.1016/j.nbt.2016.05.001>
- Laycock B, Halley P, Pratt S, Werker A, Lant P (2013) The chemomechanical properties of microbial polyhydroxyalkanoates. *Prog Polym Sci* 38(3–4):536–583. <https://doi.org/10.1016/j.progpolymsci.2012.06.003>
- Laycock B, Arcos-Hernandez MV, Langford A, Pratt S, Werker A, Halley PJ et al (2014) Crystallisation and fractionation of selected polyhydroxyalkanoates produced from mixed cultures. *N Biotechnol* 31(4):345–356. <https://doi.org/10.1016/j.nbt.2013.05.005>
- Levett I, Birkett G, Davies N, Bell A, Langford A, Laycock B et al (2016) Techno-economic assessment of poly-3-hydroxybutyrate (PHB) production from methane—the case for thermophilic bioprocessing. *J Environ Chem Eng* 4:3724–3733
- Li SY, Dong CL, Wang SY, Ye HM, Chen G-Q (2011) Microbial production of polyhydroxyalkanoate block copolymer by recombinant *Pseudomonas putida*. *Appl Microbiol Biotechnol* 90(2):659–669. <https://doi.org/10.1007/s00253-010-3069-2>
- López JC, Quijano G, Pérez R, Muñoz R (2014) Assessing the influence of CH₄ concentration during culture enrichment on the biodegradation kinetics and population structure. *J Environ Manage* 146:116–123. <https://doi.org/10.1016/j.jenvman.2014.06.026>
- Luangthongkam P (2019) Biosynthesis of Polyhydroxyalkanoates (PHAs) in methane-utilizing mixed cultures. Ph.D. Thesis, The University of Queensland
- Luangthongkam P, Laycock B, Evans P, Lant P, Pratt S (2019) Thermophilic production of poly(3-hydroxybutyrate-co-3-hydrovalerate) by a mixed methane-utilizing culture. *N Biotechnol* 53:49–56. <https://doi.org/10.1016/j.nbt.2019.06.008>
- Matsen JB, Yang S, Stein LY, Beck D, Kalyuzhnaya MG (2013) Global molecular analyses of methane metabolism in methanotrophic alphaproteobacterium, *Methylosinus trichosporium* OB3b. Part I: transcriptomic study. *Front Microbiol* 4:40. <https://doi.org/10.3389/fmicb.2013.00040>
- Morgan-Sagastume F, Karlsson A, Johansson P, Pratt S, Boon N, Lant P et al (2010) Production of polyhydroxyalkanoates in open, mixed cultures from a waste sludge stream containing high levels of soluble organics, nitrogen and phosphorus. *Water Res* 44(18):5196–5211. <https://doi.org/10.1016/j.watres.2010.06.043>
- Myung J, Galega WM, Van Nostrand J, Yuan T, Zhou J, Criddle C (2015) Long-term cultivation of a stable *Methylocystis*-dominated methanotrophic enrichment enabling tailored production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate). *Biores Technol* 198:811–818. <https://doi.org/10.1016/j.biortech.2015.09.094>
- Myung J, Flanagan JCA, Waymouth RM, Criddle CS (2016) Methane or methanol-oxidation dependent synthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by obligate type II methanotrophs. *Process Biochem* 50(5):561–567. <https://doi.org/10.1016/j.procbio.2016.02.005>
- Myung J, Flanagan JCA, Waymouth RM, Criddle CS (2017) Expanding the range of polyhydroxyalkanoates synthesized by methanotrophic bacteria through the utilization of omega-hydroxyalkanoate co-substrates. *AMB Express* 7:118. <https://doi.org/10.1186/s13568-017-0417-y>
- Pfluger AR, Wu WM, Pieja AJ, Wan J, Rostkowski KH, Criddle CS (2011) Selection of Type I and Type II methanotrophic proteobacteria in a fluidized bed reactor under non-sterile conditions. *Bioresour Technol* 102(21):9919–9926. <https://doi.org/10.1016/j.biortech.2011.08.054>
- Pieja AJ, Rostkowski KH, Criddle CS (2011a) Distribution and selection of poly-3-hydroxybutyrate production capacity in methanotrophic proteobacteria. *Microb Ecol* 62(3):564–573. <https://doi.org/10.1007/s00248-011-9873-0>

- Pieja AJ, Sundstrom ER, Criddle CS (2011b) Poly-3-hydroxybutyrate metabolism in the type II methanotroph *Methylocystis parvus* OBBP. *Appl Environ Microbiol* 77(17):6012–6019. <https://doi.org/10.1128/AEM.00509-11>
- Pieja AJ, Sundstrom ER, Criddle CS (2012) Cyclic, alternating methane and nitrogen limitation increases PHB production in a methanotrophic community. *Biores Technol* 107:385–392. <https://doi.org/10.1016/j.biortech.2011.12.044>
- Qi Q, Rehm BH (2001) Polyhydroxybutyrate biosynthesis in *Caulobacter crescentus*: molecular characterization of the polyhydroxybutyrate synthase. *Microbiology* 147(Pt 12):3353–3358. <https://doi.org/10.1099/00221287-147-12-3353>
- Ratcliff WC, Kadam SV, Denison RF (2008) Poly-3-hydroxybutyrate (PHB) supports survival and reproduction in starving rhizobia. *FEMS Microbiol Ecol* 65(3):391–399. <https://doi.org/10.1111/j.1574-6941.2008.00544.x>
- Rostkowski KH, Criddle CS, Lepech MD (2012) Cradle-to-gate life cycle assessment for a cradle-to-cradle cycle: biogas-to-bioplastic (and back). *Environ Sci Technol* 46(18):9822. <https://doi.org/10.1021/es204541w>
- Rostkowski KH, Pfluger AR, Criddle CS (2013) Stoichiometry and kinetics of the PHB-producing type II methanotrophs *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP. *Biores Technol* 132:71–77. <https://doi.org/10.1016/j.biortech.2012.12.129>
- Salehizadeh H, Van Loosdrecht MCM (2004) Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance. *Biotechnol Adv* 22(3):261–279. <https://doi.org/10.1016/j.biotechadv.2003.09.003>
- Shah NN, Hanna ML, Taylor RT (1996) Batch cultivation of *Methylosinus trichosporium* OB3b: V. Characterization of poly-β-hydroxybutyrate production under methane-dependent growth conditions. *Biotechnol Bioeng* 49(2):161–171
- Silva LF, Gomez JGC, Oliveira MS, Torres BB (2000) Propionic acid metabolism and poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P3HB-co-3HV) production by *Burkholderia* sp. *J Biotechnol* 76(2):165–174. [https://doi.org/10.1016/S0168-1656\(99\)00184-4](https://doi.org/10.1016/S0168-1656(99)00184-4)
- Slater S, Houmiel KL, Tran M, Mitsky TA, Taylor NB, Padgett SR et al (1998) Multiple β-ketothiolases mediate poly(β-hydroxyalkanoate) copolymer synthesis in *Ralstonia eutropha*. *J Bacteriol* 180(8):1979–1987
- Solaiman DKY, Swingle BM (2010) Isolation of novel *Pseudomonas syringae* promoters and functional characterization in polyhydroxyalkanoate-producing pseudomonads. *N Biotechnol* 27(1):1–9. <https://doi.org/10.1016/j.nbt.2009.12.003>
- Stein LY, Bringel F, DiSpirito AA, Han S, Jetten MSM, Kalyuzhnaia MG et al (2011) Genome sequence of the methanotrophic alphaproteobacterium *Methylocystis* sp. Strain Rockwell (ATCC 49242). *J Bacteriol* 193(10):2668–2669
- Strong P, Laycock B, Mahamud S, Jensen P, Lant P, Tyson G et al (2016) The opportunity for high-performance biomaterials from methane. *Microorganisms* 4:11
- Sudesh K, Abe H, Doi Y (2000) Synthesis, structure, and properties of polyhydroxyalkanoates- biological polyesters. *Prog Polym Sci* 25:1503–1555
- Sundstrom ER, Criddle CS (2015) Optimization of methanotrophic growth and production of poly(3-Hydroxybutyrate) in a high-throughput microbioreactor system. *Appl Environ Microbiol* 81(14):4767–4773. <https://doi.org/10.1128/aem.00025-15>
- Takeguchi M, Okura I (2000) Role of iron and copper in particulate methane monooxygenase of *Methylosinus trichosporium* OB3b. *Catal Surv Jpn* 4(1):51–63. <https://doi.org/10.1023/A:1019036105038>
- Tan GY, Chen CL, Li L, Ge L, Wang L, Razaad I et al (2014) Start a research on biopolymer polyhydroxyalkanoate (PHA): a review. *Polymers* 6(3):706–754. <https://doi.org/10.3390/polym6030706>
- Thakor NS, Patel MA, Trivedi UB, Patel KC (2003) Production of poly(β-hydroxybutyrate) by *Comamonas testosteroni* during growth on naphthalene. *World J Microbiol Biotechnol* 19(2):185–189. <https://doi.org/10.1023/a:1023295009846>
- Tripathi L, Wu LP, Chen J, Chen GQ (2012) Synthesis of diblock copolymer poly-3-hydroxybutyrate-block-poly-3-hydroxyhexanoate [PHB-b-PHHx] by a β-oxidation weakened *Pseudomonas putida* KT2442. *Microb Cell Fact* 11(1):44. <https://doi.org/10.1186/1475-2859-11-44>
- Urakami T, Komagata K (1987) Characterization and identification of methanol-utilizing *Hyphomicrobium* strains and a comparison with species of *Hyphomonas* and *Rhodomicrobium*. *J Gen Appl Microbiol* 33(6):521–542. <https://doi.org/10.2323/jgam.33.521>
- Wang Y, Liu S (2014) Production of (R)-3-hydroxybutyric acid by *Burkholderia cepacia* from wood extract hydrolysates. *AMB Express* 4:28. <https://doi.org/10.1186/s13568-014-0028-9>
- Xin J, Zhang Y, Dong J, Song H, Xia C (2011) An experimental study on molecular weight of poly-3-hydroxybutyrate (PHB) accumulated in *Methylosinus trichosporium* IMV 3011. *Afr J Biotechnol* 10(36):7078–7087
- Yang S, Matsen JB, Konopka M, Green-Saxena A, Clubb J, Sadilek M et al (2013) Global molecular analyses of methane metabolism in methanotrophic alphaproteobacterium, *Methylosinus trichosporium* OB3b. Part II metabolomics and ¹³C-labeling Study. *Front Microbiol*. <https://doi.org/10.3389/fmicb.2013.00070>
- Zhang T, Wang X, Zhou J, Zhang Y (2017) Enrichments of methanotrophic-heterotrophic cultures with high poly-β-hydroxybutyrate (PHB) accumulation capacities. *J Environ Sci* 65:133–143. <https://doi.org/10.1016/j.jes.2017.03.016>
- Zhao S, Fan C, Hu X, Chen J, Feng H (1993) The microbial production of polyhydroxybutyrate from methanol. *Appl Biochem Biotechnol Part A Enzyme Eng Biotechnol* 39(1):191–199. <https://doi.org/10.1007/bf02918989>
- Zhu C, Nomura CT, Perrotta JA, Stipanovic AJ, Nakas JP (2010) Production and characterization of poly-3-hydroxybutyrate from biodiesel-glycerol by *Burkholderia cepacia* ATCC 17759. *Biotechnol Prog* 26(2):424–430. <https://doi.org/10.1002/btpr.355>
- Zuniga C, Morales M, Le Borgne S, Revah S (2011) Production of poly-beta-hydroxybutyrate (PHB) by *Methylobacterium organophilum* isolated from a methanotrophic consortium in a two-phase partition bioreactor. *J Hazard Mater* 190(1–3):876–882. <https://doi.org/10.1016/j.jhazmat.2011.04.011>

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Submit your manuscript to a SpringerOpen® journal and benefit from:

- Convenient online submission
- Rigorous peer review
- Open access: articles freely available online
- High visibility within the field
- Retaining the copyright to your article

Submit your next manuscript at ► [springeropen.com](https://www.springeropen.com)