Methanotrophic production of polyhydroxybutyrate-co-hydroxyvalerate with high hydroxyvalerate content

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Abstract

Type II methanotrophic bacteria are a promising production platform for PHA biopolymers. These bacteria are known to produce pure poly-3-hydroxybutyrate homopolymer (PHB). We isolated a strain, *Methylocystis* sp. WRRC1, that was capable of producing a wide range of polyhydroxybutyrate-co-hydroxyvalerate copolymers (PHB-co-HV) when co-fed methane and valerate or n-pentanol. The ratio of HB to HV monomer was directly related to the concentration of valeric acid in the PHA accumulation media. We observed increased incorporation of HV and total polymer under copper-free growth conditions. The PHB-co-HV copolymers produced had decreased melting temperatures and crystallinity compared with methanotroph-produced PHB.

1. Introduction

Polyhydroxyalkanoates (PHA) are biologically produced, biodegradable polymers with properties similar to polypropylene; these properties can be changed by varying the copolymer content of the polymer [1–3]. Many different bacterial species accumulate PHA as a carbon and/or energy storage polymer when subjected to conditions of carbon excess and/or nutrient deficiency [1,3]. PHA commercialization has been limited to date, in part due to the cost of the feedstock (typically sugars such as glucose and sucrose) used to produce the PHA, which may account for up to 30% of the total production cost [4]. One method of reducing production costs is to utilize a waste substrate as a feedstock; methane gas is one such feedstock that is often considered a waste and is produced as biogas through anaerobic digestion of waste at facilities including landfills, wastewater treatment plants, and agricultural operations. This biogas is often flared due to insufficient incentives for its capture and use. It has been estimated that 466 billion cubic feet of methane are released annually from landfills alone in the United States [5].

Type II methanotrophs, which utilize the serine cycle for carbon assimilation, are known to produce poly-hydroxybutyrate (PHB), the most common PHA, under conditions of nutrient deficiency [6–11]. Asenjo (1986) reported PHB accumulation of up to 68% of the total dry cell weight in *Methylocystis parvus* OBBP, although the total concentration of PHB was low (<2 g/L) [6]. Wendlandt (2001) achieved PHB levels of up to about 30 g/L in a non-sterile culture dominated by *Methylocystis* sp. GB 25 [10].

Most methanotrophs have not been shown to utilize any substrate other than methane [12][13]. Although PHB is the most commonly produced PHA, the material properties of pure PHB may not be ideal for many applications. PHB is relatively brittle, and its melting temperature is close to the temperature at which degradation begins, which creates a relatively narrow thermal processing window and can make polymer processing difficult. Degradation may begin at 200°C [14] and the melting temperature can range from 160-200°C, although it is most

commonly reported from 170-180°C [3,14–16]. The melting and decomposition temperatures vary with polymer purity and molecular weight [17].

PHB copolymers have improved mechanical properties and have a wider thermal processing window due to a lower melting temperature. Although reported melting temperatures vary between studies, the melting temperature of PHB-co-HV has been shown to decrease with increasing HV content up to 40%, with PHB-co-40% HV having a melting temperature around 65°C [18]. According to another review, the melting temperature of PHB-co-HV copolymers decreases with increasing HV fraction and ranges from 179°C (pure PHB) to 170°C (3 mol% HV) to 137°C (25 mol% HV) [3]. PHB-co-HV copolymers have been shown to be produced by the methylotrophic bacteria *Methylobacterium organophilum* CZ-2 from propionate and citrate with methane as a co-substrate [19] as well as by a mixed culture containing 65-77% methanotrophic bacteria fed methane and valerate [20].

Young's modulus decreases with increasing HV content in PHB-co-HV copolymers (3.5 for pure PHB to 0.7 GPA for PHB-co-25% HV), as does tensile strength (40 MPa for pure PHB to 30 MPa for PHB-co-25% HV) [3]. The notched izod impact method shows that impact strength increases with increasing copolymer content (from 50 J/m for pure PHB to 400 J/m for PHB-co-25% HV), which illustrates that the samples become less brittle with increasing HV content [3].

This work explores the incorporation of 5 carbon co-substrates, valerate and pentanol, into PHA polymers by a pure strain of type II methanotrophic bacteria. We find that both valerate and pentanol, when co-fed with methane, are able to support the production of PHB-co-HV in a strain that produces pure PHB on methane alone.

2. Methods

2.1. Bacterial strains and growth conditions

A pure strain was isolated from a mixed methanotrophic culture of *Methylocystis parvus* OBB3. The 16S rRNA sequence (GenBank KT827743) was determined as previously described[21]. BLAST analysis[22] demonstrated that the 16S rRNA sequence had >99.5% identity to multiple *Methylocystis* strains. Therefore, this strain was designated *Methylocystis* sp. WRRRC1. Cells were grown in W1 mineral media [23]. Copper-free growth media was the same but omitted copper.

Biomass for PHA production was generated in 2 L fernbach flasks with airtight septa containing 500 mL W1. Fernbachs were flushed with a 1:1 mixture of methane and oxygen for two minutes and quickly sealed. All cultures were shaken at 300 RPM at 30°C. Cultures were routinely plated on rich nutrient agar to verify purity. Seed cultures were grown to an optical density of ~1 and centrifuged at 2000 x g for 20 minutes. Cells were re-suspended in nitrate-free media to desired optical density. For valerate supplementation, 310 g/L sodium valerate, pH 7 was added to media to desired valerate concentration after re-suspension in nitrogen free media; n-pentanol was added directly to the media to desired concentration. For serum bottle experiment, 10 mL of culture re-suspended in nitrogen free media to an OD of ~3 were added to 160 mL serum bottles, which were sealed with butyl stoppers (Geo-microbial Technologies), evacuated, and gassed with 75 mL methane and 75 mL oxygen (Praxair). Gas-tight box experiments were conducted using the EnzyScreen system [24] (www.enzyScreen.com) with 3 mL of re-suspended culture added to each well of a 24 well pyramid bottom deepwell plate (EnzyScreen, CR1424C). Plates were covered with a sandwich cover (EnzyScreen, CR1224) and clamped inside the gas tight box (EnzyScreen, CR1601). The box was flushed with 1.7 L/minute methane and 1 L/minute oxygen for five minutes.

Yields of PHA formation on methane and co-substrates were calculated as the weight of PHA produced divided by the total amount of substrate consumed (g/g). Tukey HSD test was calculated using the agricolae package in R.

2.2. Purification and production of solvent-cast PHB and PHB-co-HV films

Cells were grown in 24 plates as above for 72 hours, and 36 mL of culture pooled from 12 wells was centrifuged at 3645 x g for 10 minutes. Cells pellets were re-suspended in 25 mL of 70% ethanol and incubated at room temperature for 15 minutes with occasional agitation. This suspension was centrifuged for 15 minutes at 1620 X g and re-suspended in 20 mL 99% acetone (Sigma-Aldrich, MO, USA). After 30 minutes, samples were centrifuged at 3645 x g; the supernatant was removed and pellets were dried under a stream of air for one hour. Dried pellets were re-suspended in 3 mL chloroform and incubated at 60° C for 90 minutes with periodic shaking to dissolve the polymer. The suspension was filtered through a glass fiber filter; the filtrate was evaporated overnight under a stream of air in a fume hood.

2.3. PHB-co-HV quantification

Polyhydroxyalkanoates were analyzed by gas-chromatography as described [25,26] with modifications. Lyophilized cells were suspended in 1.0 mL of chloroform in a 13 x 100 mm screw cap tube and 1.0 mL of methanol containing 15% (v/v) concentrated sulphuric acid was added. The tubes were sealed and heated at 100-105°C for 90-100 min with intermittent shaking. After cooling, 1.5 mL of dH₂O was added and the tubes were vortexed for 10-15 sec. After separation, the organic layer was transferred to a microcentrifuge tube containing approximately 50 mg of anhydrous magnesium sulfate, sealed, shaken and then centrifuged at 14,000 x g in a microcentrifuge for 5 min. The supernatant was then transferred to

autosampler vials. Analysis of samples was conducted on an Agilent 6890 GC equipped with a 30 m, JW -5ht column and FID detector with helium as the carrier gas. The inlet temperature was 200°C and the detector temperature was 220°C. The oven temperature was initially 50°C for 2 minutes followed by a ramp to 195°C at 8°C per minute. PHB (Sigma) and PHB-co-PHV (Sigma) were used as standards, and benzoic acid dissolved in methanol was used as an internal standard. Peak integration was obtained using the Agilent Chem Station software (Agilent).

2.4. Valerate quantification

Analysis of valerate concentrations was performed with an HPLC system (Agilent, San Jose, CA) equipped with a quaternary pump, an autosampler fitted with a 100 µL loop, and a refractive index detector (RID). The analyses were performed isocratically at 0.5 mL/min and 40 °C with a 300 X 7.8 mm i.d. cation exchange column (Aminex HPX-87H) equipped with a cation H⁺ microguard cartridge (Bio-Rad Laboratories, Hercules, CA). The mobile phase was 0.01 N H₂SO₄, prepared by diluting reagent grade sulfuric acid with distilled water, filtering through a 0.45 µm membrane filter, and degassing under vacuum. Analytical grade reagents were used as standards (Sigma Chemical).

2.5. Pentanol quantification

N-Propanol content was measured on an Agilent 7890 GC-FID equipped with an RTX-2330 (30mx0.25mmx0.2µm) column. Samples were centrifuged to remove cell debris and 0.5 µL of supernatant was injected directly into the machine with a 4:1 split and an initial temperature of 55°C for 2min. After the initial hold, the oven was ramped at 2.5°C/min to 60°C and then to 180°C at 30°C/min. Samples were compared to a standard curve of n-pentanol in water.

2.6. FT-IR

Films were measured on a Nicolet i10 FTIR equipped with a diamond ATR. For each sample, 64 scans were averaged from 650 to 4000 cm^{-1} at a resolution of 4 cm^{-1} . Spectra were normalized using OMNIC Spectra software (Thermo Scientific, MA, USA).

2.7. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed on a Perkin Elmer DSC 8000. Reference temperature ranged from -50°C to 220°C with a ramp of 5°C per minute. Pyrus software was used to calculate melting temperature and enthalpy of melting. The degree of crystallinity, H^* , was determined according to the formula $H^* = (H_f - H_{100\%})/100$. The heat of enthalpy of 146 J/g was used as $H_{100\%}$ [27].

3. Results and Discussion

3.1. Mass balance of PHA production with valerate and n-pentanol co-substrates

To examine copolymer production in the presence of co-substrates valerate and n-pentanol, cells were resuspended in nitrogen free media to a density of 1 g/L and fed 1 g/L valerate or n-pentanol. Control cells were fed methane alone. No growth was observed in cultures fed valerate or n-pentanol in the absence of methane. Both co-substrates supported the production of PHB-co-HV, while control cells produced only PHB. The yield of PHA was highest in cells fed valerate and methane (Table 1). PHA yield on n-pentanol and methane were slightly higher to yields on methane alone. Methane consumption was similar in the methane control and valerate plus methane conditions. Inclusion of pentanol in the media greatly reduced methane consumption. Valerate increased total PHA yield without inhibiting methane consumption and was examined further as a co-substrate for the production of PHB-co-HV from

methane. Total biomass increased by 0.8 g/L in valerate and methane samples, compared to 0.5 g/L in both pentanol and methane and the methane only control.

3.2. Valerate concentration and PHB-co-HV production

Copper is an essential co-factor for production of the particulate methane monooxygenase. In the absence of copper, some methanotrophs oxidize methane using a copper-independent soluble methane monooxygenase[28]. To investigate the impact of soluble monooxygenase on copolymer production, cultures of *Methylocystis* sp. WRRRC1 resuspended in nitrogen free media to a density of 3 g/L with sodium valerate concentrations ranging from 0.03% to 1.3% were tested to determine the range of copolymer production. When valerate was absent from the media supplemented with copper, the polymer consisted solely of hydroxybutyrate (Fig. 1a). The fraction of hydroxyvalerate in the polymer increased with increasing valerate concentrations, up to 0.34%, at which concentration cells produced 50% HV in the copolymer. At 0.03% valerate, the copolymer produced contained 15% HV. Inclusion of valerate in the media not only increased the amount of HV in the copolymer, but also the total amount of PHA polymer produced by the culture. In cells grown without valerate, PHA accounted for roughly 30% of cell dry weight; cells grown in the presence of 0.34% valerate accumulated 60% PHA. This indicates that the accumulation of PHA in methanotrophs is dictated by metabolic availability of precursors for PHA synthesis and not likely limited by the activity of PHA-forming enzymes.

A threshold effect was observed at high valerate concentrations; valerate concentrations of 0.7% and above were inhibitory to both hydroxyvalerate incorporation and PHA production in general (Fig. 1a). Cultures incubated with valerate above the threshold accumulated 15% or less PHA, compared with 30% for cultures with no valerate added to the

media. Cells grown above the valerate threshold did not incorporate valerate into the polymer, which was made up solely of HB.

3.3. Influence of copper on PHB-co-HV production

Cells grown and re-suspended in copper-free media to a density of 3 g/L showed an increase in HV incorporation compared to copper grown cells (Fig. 1). The maximum mole fraction HV for cells grown in copper-free media was 58% compared to 50% for copper grown cultures. Growth in copper-free media also increased the maximum overall PHB-co-HV production in the methanotrophs to 78%, compared with 60% for methanotrophs cultured in the presence of copper. This finding is unexpected; copper-free methanotroph cultures have been shown to accumulate biomass at lower yields than cultures grown on copper[28–30].

The valerate concentration threshold for inhibition of PHA accumulation remained the same in copper-free cultures; however, the degree of inhibition by high valerate was diminished. In contrast to copper-grown cultures, copper-free cells still incorporated HV in the polymer at high valerate concentrations. Though the mole fraction HV remained high beyond the inhibition threshold in copper-free cultures, the amount of total polymer was greatly diminished. However, it is possible that the metabolic shift during nitrogen deprivation is altered when cultures are grown in media without copper.

3.4. Dynamics of PHB-co-HV synthesis and valerate uptake

In order to investigate the dynamics of methanotrophic PHB-co-HV synthesis in the presence of 0.14% valerate in the presence of copper, polymer amount, composition, and valerate uptake were measured over a period of 70 hours after the induction of PHA production by nitrogen deprivation. Prior to nitrogen deprivation, cells were grown in the absence of valerate. Cells were re-suspended to a density of 0.9 g/L and contained less than 1% PHB (g/g) before transfer into nitrogen free media. The mole fraction HV in the polymer increased from

23% after 24 hours to 43% after 70 hours (Fig. 2a). The rate of HV incorporation into the polymer was greatest between 24 and 48 hours (Fig. 2b). Between 48 and 70 hours, the rate of HB incorporation slowed dramatically, while the rate of HV incorporation remained higher than during the first 24 hours. Valerate steadily decreased in the culture media throughout the experiment and was nearly depleted by 70 hours (Fig. 2c). The decline in rates of HB incorporation may be due to a decline in the acetyl-CoA pool during the final phase of the stringent response [31,32]. Biomass increased by 0.66 g/L over 72 hours while PHA content increased by 0.93 g/L, indicating that some of the initial biomass was converted to PHA during nitrogen limitation.

3.5 Characterization of methanotrophic PHB and PHB-co-HV

Films made of methanotroph-produced PHB, PHB-co-15% HV, and PHB-co-43% HB were solvent cast for further characterization. FTIR spectra of PHB and copolymer samples overlap greatly, with some qualitative distinctions differentiating samples (Fig. 3). The major characteristic carbonyl stretch at $\sim 1720\text{ cm}^{-1}$ shifted to a higher wave numbers as HV percentage of the polymer increased. The asymmetry of the doublet at 1040-1060 nm, corresponding to the C-O bond in the ester group, diminished as HV levels increased [33]. Differences between PHB and copolymers were observed in the spectra at 1228 cm^{-1} , which has been reported to be sensitive to the degree of crystallinity in samples [34].

Differential scanning calorimetry was used to measure the melting temperature (T_m) of films and determine the degree of crystallinity (Fig. 4, Table 2). The T_m of PHB, PHB-co-15% HV, and PHB-co-43% HV were 180, 161, and 170°C respectively. The U-shaped response of T_m is a product of isodimorphism in the copolymer and agrees with previous reports [35,36]. In contrast to the T_m , the degree of crystallinity drops throughout the copolymer space sampled; crystallinity of PHB-co-43% HV was 5%, compared with 52% for the solvent cast PHB film.

239

240 **4. Conclusions**

241 The formation of PHB-co-HV copolymers by type II methanotrophs fed valerate and n-pentanol
242 during PHA production was studied. It was found that methanotrophs can produce PHB-co-HV
243 when fed valerate or n-pentanol during nitrogen limitation. Valerate co-feeding produced PHB-
244 co-HV with nearly 50% higher yield than that of n-pentanol co-feeding. Supplementation at
245 valerate levels as low as 0.03% resulted in significant levels of HV polymer incorporation. With
246 higher levels of valerate, the methanotrophs could produce PHB-co-HV with nearly 60% HV
247 before valerate concentrations became inhibitory. Both valerate feeding and growth on copper-
248 free media increased the overall level of PHA production in the methanotrophs to a maximum of
249 78% of cell dry weight, the highest level of PHA produced by methanotrophs that has been
250 reported to date. The incorporation of HV into the polymer remained fairly consistent, even
251 after the rate of HB incorporation fell sharply. Increasing the fraction of HV in the copolymer
252 decreased crystallinity. A U-shape response to increased HV levels was found for the melting
253 point. This study indicates improvements in both yield and polymer characteristics can be
254 gained by supplementing valerate to cells grown on an inexpensive substrate, methane.

255

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TABLES

Substrate	Cosubstrate initial (g/L)	Cosubstrate consumed (g/L)	CH ₄ consumed (g/L)	PHA-HB produced (g/L)	PHA-HV produced (g/L)	Yield PHA/ Substrate	Yield P(HV)/ cosubstrate
CH ₄			0.43 ^a	0.20	0.00 ^a	0.38 ^a	-
Pentanol + CH ₄	1.00	0.91	0.06 ^b	0.14	0.17 ^b	0.46 ^{ab}	0.38
Valerate + CH ₄	0.99	0.78	0.39 ^a	0.25	0.32 ^c	0.67 ^a	0.63

Table 1. Methane and cosubstrate consumption and PHA produced. Superscripts refer to TukeyHSD differences with p <0.05.

	Melting Temp (T _m , C)	Delta H (J/g)	% Crystallinity
PHB	180	76	52
PHB-co-15% HV	161	34	23
PHB-co-43% HV	170	7.3	5

Table 2. Thermal physical characteristics of methanotrophs PHB and PHB-co-HV

FIGURE LEGENDS

Figure 1. Percent PHA and mol. percent HV at varying sodium valerate concentrations. Cells were grown and resuspended in either the (a) presence or (b) absence of copper.

Figure 2. Time course of PHB-co-HV production. (a) Percent total PHA of cell dry weight and mol percent HV from 0 to 70 hours. (b) Rate of increase of HB and HV units in the polymer across three time periods. (c) Valerate levels in culture supernatant from 0 to 70 hours.

Figure 3. Fourier-transform infrared spectra of PHA with varying levels of HV.

Figure 4. Differential scanning calorimetry of PHA with varying levels of HV.

Fig1a

Fig1b

Fig2a

Fig2b

Fig2c

Fig3

Fig4