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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.

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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 to 200° C, although it is most commonly reported from 170 to 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].

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

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

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. WRRC1. 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 × *g* for 20 min. 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/min methane and 1 L/min 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 agricolea 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 h, and 36 mL of culture pooled from 12 wells was centrifuged at 3645 × *g* for 10 min. Cells pellets were re-suspended in 25 mL of 70% ethanol and incubated at room temperature for 15 min with occasional agitation. This suspension was centrifuged for 15 min at 1620 × *g* and re-suspended in 20 mL 99% acetone (Sigma–Aldrich, MO, USA). After 30 min, samples were centrifuged at 3645 × *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 min 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 × 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 dH$_2$0 was added and the tubes were vortexed for 10–15 s. 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 × 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 −5 ht 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 min 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 × 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 H_2SO_4, 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 (30 m × 0.25 mm × 0.2 μm) column.