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Biogas-based polyhydroxyalkanoates production by *Methylocystis hirsuta*: A step further in anaerobic digestion biorefineries

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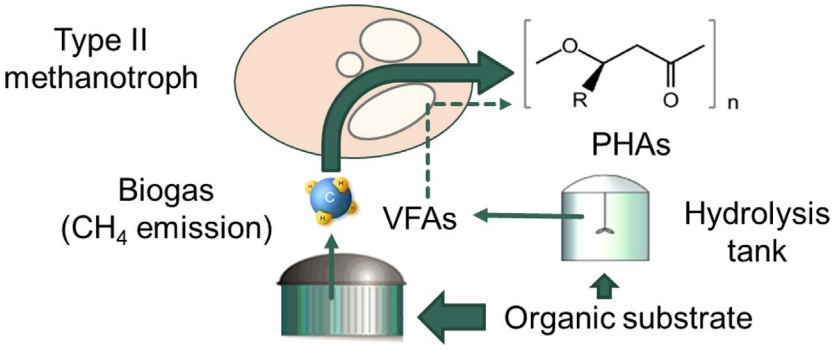
GRAPHICAL ABSTRACT



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ABSTRACT

The potential of biogas (with and without H_2S) and volatile fatty acids (VFAs) to support microbial growth and accumulation of polyhydroxyalkanoates (PHAs) in type II methanotrophs was evaluated batchwise under aerobic conditions. *Methylocystis hirsuta* was able to grow on artificial biogas (70% CH_4, 29.5% CO_2, 0.5% H_2S) and accumulate PHA up to 45 ± 1% (wt.%) under N-limited conditions. The presence of CO_2 and H_2S did not significantly influence the growth and PHA synthesis in *M. hirsuta* compared to control tests provided with pure CH_4 at similar concentrations. Likewise, the addition of VFAs to the cultivation broth at initial concentrations of 100–200 mg L^{-1} did not hamper the growth of this strain on artificial biogas. Indeed, the addition of 10% extra carbon in the form of individual VFAs resulted in an increase in the maximum PHA yield and final PHA content up to 0.45–0.63 gPHA gSubstrate ^{1} and 48–54% (wt.%), respectively, at the expense of a higher energy demand. Valeric acid supplementation supported the highest 3-hydroxyvalerate content (13.5%) within the biocomposite. In this context, this study demonstrated for the first time that 3-hydroxyvalerate synthesis by *M. hirsuta* did not depend on CH_4 assimilation.

1. Introduction

Methane (CH_4), which accounts for 10–16% of the global warming impact worldwide, represents nowadays the second most important greenhouse gas. In nature, CH_4 is mainly emitted from the anaerobic decomposition of organic matter in wetlands and oceans. However, more than 60% of CH_4 emissions worldwide are anthropogenic [1–3]. Waste and wastewater treatment plants (WWTPs) represent one of the most significant emission sources of CH_4 (20,000 ktons CO_2-eq in 2014 in the EU-28), which is often released in the form of a biogas typically

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composed of 50–70% CH_4, 30–50% CO_2 and 0–0.5% H_2S (*v/v*) [4,5]. Anaerobic digesters in such facilities process different types of organic feedstock, liquid and solid waste, while producing i) sludge that can be used as an agricultural fertilizer and ii) biogas to be employed for electricity and/or heat production. In this regard, the European Biogas Association (EBA) report claimed that by the end of 2013 more than 14,000 anaerobic digesters were in service in Europe with at least 7400 MW of electricity generation capacity [6]. However, despite the potential of biogas as a renewable energy source for heat and electricity generation, the high investment costs needed for on-site energy recovery or the high costs associated to biomethane production ($1.08 € Nm^{-3}$ in the EU market compared to 0.30–0.67 $€ Nm^{-3}$ for natural gas) promote biogas flaring or venting to the atmosphere in low-medium size facilities [7,8]. In addition, the huge reserves of shale gas worldwide, along with its affordable extraction costs, do not forecast a scenario of increased natural gas prices (where biogas could advantageously compete). In this context, the development of cost-effective technologies for the bioconversion of biogas into high-added value products could eventually mitigate biogas emissions from waste/wastewater treatment facilities along with the implementation of anaerobic digestion as a platform for organic pollution control.

Polyhydroxyalkanoates (PHAs), such as poly-3-hydroxybutyrate (PHB), poly-3-hydroxyvalerate (PHV) and their copolymer (PHBV), are polyesters biologically produced under unbalanced nutrient conditions (e.g. N limitation). PHAs have the potential to substitute conventional plastics such as polyethylene or polypropylene due to their biocompatibility, biodegradability and their versatile thermal and mechanical properties. The market price of PHAs ranges from 4 to $20 € kg_{PHA}^{-1}$, which greatly depends on the monomer composition of the biocomposite, the carbon source, the microbial strain used and the product purity [9]. Despite its rapid decrease in the past 5 years, the market price of PHAs is still higher than that of fossil-based polyesters due to the high costs of biopolymer downstreaming and carbon source acquisition, the later accounting for 30–40% of the final PHA price [8,9]. In this regard, CH_4 has recently emerged as a low-cost and environmentally friendly feedstock for PHA production [10,11]. To the best of the authors' knowledge, most studies reported to date on methanotrophic PHA production have been mainly focused on the use of pure CH_4 or natural gas as substrate [12–14]. Controversy still exists in literature about the technical and microbiological feasibility of biogas (containing the toxic and acid gases CO_2 and H_2S) as a feedstock for PHA production [12,15]. Moreover, the direct addition to the methanotrophic cultivation broth of volatile fatty acids (VFAs), which are readily available during anaerobic digestion, could increase PHA yields and tailor the composition of the biocomposite during biogas bioconversion. However, the few studies reported to date restrict the use of VFAs to their corresponding salts (i.e. sodium valerate, sodium propionate or sodium 3-hydroxybutyrate), which overcome the pH-associated effects of VFAs but hinder their applicability within this biorefinery concept. In this context, neither the potential of biogas nor the influence of VFA supplementation on PHA accumulation by methanotrophs have been yet systematically addressed [16–19]. A successful bioconversion of biogas into VFA-tailored biopolymers would represent the cornerstone of a new generation of biogas biorefineries supporting a low-cost and environmentally friendly conversion of residual organic matter into multiple high-added value products.

This study aimed at evaluating the feasibility of artificial biogas as a feedstock to support the growth of the type II methanotroph *Methylocystis hirsuta* coupled to the synthesis of PHAs. Additionally, the potential of acetic, butyric, propionic and valeric acids to support *M. hirsuta* growth and modify the composition of the biogas-based PHA biocomposite was here evaluated for the first time.

2. Materials and methods

2.1. Strain, chemicals and culture conditions

The methanotrophic strain *Methylocystis hirsuta* was acquired from DSMZ culture collection (DSM No. 18500, Leibniz Institute, Germany). This type II methanotroph was selected based on i) its ability to produce PHB from CH_4 through the serine pathway and ii) the fact that the highest PHA contents up to date have been recorded for this strain [13]. Synthetic biogas (70% CH_4, 29.5% CO_2, 0.5% H_2S), CH_4 (≥99.5%), He (≥99.5%), O_2 (≥99.5%) and CO_2 (≥99.9%) were purchased from Abelló Linde S.A. (Barcelona, Spain). Poly[(R)-3-hydroxybutyric acid-*co*-(R)-3-hydroxyvaleric acid] (molar ratio 88/12, ≥99.99%), valeric acid (≥99%) and butyric acid (≥99%) were obtained from Sigma-Aldrich® (Sigma-Aldrich, St. Louis, USA). Acetic acid (≥99%) was purchased from Cofarcas S.A. (Burgos, Spain). Additional reagents and chemicals were purchased from Panreac® (Barcelona, Spain) with a purity of at least 99%.

Balanced growth cultures were cultivated in Whittenbury nitrate mineral salt (NMS) medium (pH of 6.8) [20]. NMS medium supplemented with agar at 1.5% (*w/v*) was used to test culture purity along the experiment. In contrast, unbalanced growth cultures devoted to accumulate PHAs were incubated in a nitrate-free Whittenbury mineral salt medium (NFMS).

2.2. Experimental procedures

2.2.1. Inocula

M. hirsuta inocula were prepared in 125-mL serum bottles capped with butyl-rubber stoppers and crimp-sealed under a CH_4:O_2 headspace (35:65% *v/v*) and sterile conditions (Fig. 1). The serum bottles contained 50 mL of NMS inoculated at 10% (*v/v*) and were incubated in an orbital shaker at 250 rpm and 25 °C for 7 days, which entailed five CH_4:O_2 headspace renewals. The final optical density of the cultures at 600 nm (OD_{600}) was 4.0 ± 0.4 (total suspended solid concentration – TSS – of 1690 ± 169 $mg\,L^{-1}$). Unless otherwise specified, this inoculum was used for Test Series 1–4.

2.2.2. Test Series 1: Influence of artificial biogas on M. hirsuta growth

The ability of *M. hirsuta* to grow on artificial biogas (with and without H_2S) was assessed in triplicate in 2.15-L serum bottles capped with butyl-rubber stoppers and aluminium crimp seals under three different O_2-supplemented headspace atmospheres (*v/v*): H_2S-free biogas (CH_4:O_2:CO_2:He at 31.5:55.0:13.27:0.23%), biogas (CH_4:O_2:CO_2:H_2S at 31.5:55.0:13.27:0.23%) and control (CH_4:O_2:He at 31.5:55.0:13.5%). The headspace mixtures were prepared in 25 L-Tedlar bags (Sigma-Aldrich®, St. Louis, USA) using the appropriate volumes of each gas component from the cylinders and further pumped into the corresponding bottles in order to completely flush the air atmosphere out. The cultures, which contained 400 mL of NMS inoculated at 3% (*v/v*) (initial OD_{600} of 0.13 ± 0.01, corresponding to 55 ± 2 $mg\,TSS\,L^{-1}$), were magnetically stirred at 300 rpm (Multipoint 15 Variomag, Thermo Fisher Scientific, Bartlesville, USA) and 25.0 ± 0.5 °C in a temperature-controlled room. Abiotic controls for the three headspace mixtures were also prepared as above described to rule out any potential CH_4 removal due to adsorption or photolysis.

2.2.3. Test Series 2: Influence of artificial biogas on PHA synthesis by M. hirsuta

M. hirsuta was initially grown as above described in 2.15-L serum bottles containing 400 mL of NMS inoculated at 3% (*v/v*) under a CH_4:O_2:CO_2:H_2S atmosphere (31.5:55.0:13.27:0.23%) for 9–12 days (to completely deplete CH_4 from the headspace). The methanotrophic biomass was harvested by centrifugation (10,000 rpm, 8 min) and resuspended in NFMS. Then, the ability of biogas-grown *M. hirsuta* to accumulate PHAs was assessed in triplicate in 2.15-L serum bottles