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

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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.

Keywords

Biorefinery, methane, methanotroph, polyhydroxybutyrate, polyhydroxyvalerate, volatile fatty acid.

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 (20000 ktons CO_2-eq in 2014 in the EU-28), which is often released in the form of a biogas typically 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 14000 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 Institut, 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

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 (Figure 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 ºC 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.

<Fig. 1>

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 mgTSS L^{-1}), were magnetically stirred at 300 rpm (Multipoint 15 Variomag, Thermo Fisher Scientific, Bartlesville, USA) and 25.0 ± 0.5 ºC 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.

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 (10000 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 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 bottles were incubated under magnetic agitation at 300 rpm and 25.0 ± 0.5 ºC in a temperature-controlled room.

Test Series 3: Influence of the type and concentration of VFA on biogas-based M. hirsuta *growth*

M. hirsuta was grown in VFA-supplemented 125 mL serum bottles capped with butyl-rubber stoppers and crimp-sealed under sterile conditions and an artificial biogas headspace (CH_4:O_2:CO_2:H_2S at 31.5:55.0:13.27:0.23 %). The bottles, which initially contained 50 mL of NMS inoculated at 4 % (*v/v*) (initial OD_{600} of 0.15 ± 0.01,

corresponding to 65 ± 2 mgTSS L^{-1}), were incubated at 300 rpm and 25.1 ± 0.3 ºC in a temperature-controlled room. The influence of the concentration of acetic, butyric, propionic and valeric acid (named C1–C5, where C1 represented the lowest and C5 the highest VFA concentration) on *M. hirsuta* growth was evaluated in duplicate in separate batch assays. The initial concentrations of acetic acid were 92 ± 10, 197 ± 5, 243 ± 2, 324 ± 5 and 482 ± 4 mg L^{-1}, of butyric acid 68 ± 1, 139 ± 3, 182 ± 7, 273 ± 2 and 345 ± 3 mg L^{-1}, of propionic acid 64 ± 2, 123 ± 2, 214 ± 26, 258 ± 0 and 320 ± 3 mg L^{-1} and of valeric acid 57 ± 5, 114 ± 1, 177 ± 3, 238 ± 4 and 292 ± 0 mg L^{-1}. These concentrations represented 16, 31, 47, 63 and 78 % of the C initially supplied as CH_4. Abiotic controls under a CH_4:O_2:CO_2:H_2S atmosphere (31.5:55.0:13.27:0.23 %) and an initial C2 concentration for each VFA were prepared to rule out any potential CH_4 or VFA removal due to adsorption or photolysis. Individual biogas-deprived controls at an initial C2 concentration for each VFA were included in this batch assay to evaluate the ability of the strain to remove the VFA in the absence of biogas. Cosubstrate-deprived controls under a CH_4:O_2:CO_2:H_2S atmosphere (31.5:55.0:13.27:0.23 %) were also conducted.

Test Series 4: Influence of the type of VFA on biogas-based 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 again harvested by centrifugation (10000 rpm, 8 min) and resuspended in NFMS supplemented with either acetic, butyric, propionic and valeric acids at concentrations of 181 ± 16, 123 ± 2, 139 ± 1 and 130 ± 6 mg L^{-1}, respectively (corresponding to 10 % of the C initially supplied as CH_4). The bottles were finally crimp-sealed, filled with a fresh CH_4:O_2:CO_2:H_2S atmosphere

(31.5:55.0:13.27:0.23 %) and incubated at 300 rpm and 25.0 ± 0.5 ºC. Abiotic controls under a CH_4:O_2:CO_2:H_2S atmosphere (31.5:55.0:13.27:0.23 %) and/or an initial C2 concentration for each VFA were prepared to rule out any potential CH_4 or VFA removal due to adsorption or photolysis. Individual biogas-deprived controls at an initial C2 concentration for each VFA were included in this batch assay to evaluate the ability of the strain to produce PHAs in the presence of VFA without artificial biogas. Cosubstrate-deprived controls under a CH_4:O_2:CO_2:H_2S atmosphere (31.5:55.0:13.27:0.23 %) were also carried out to assess the influence of VFAs on the content and composition of the PHA synthesized.

The headspace concentration of CH_4, CO_2, O_2 and H_2S was periodically measured by GC-TCD in all test series. Liquid samples (3 mL) were periodically drawn to monitor the concentration of VFAs, PHAs, TSS and OD_{600} in all test series. Liquid samples (1 mL) were also randomly withdrawn to measure the SO_4^{2-} concentration in the liquid phase by HPLC-IC in Test Series 1 and 2. The pH of the cultivation broth was measured at the beginning and at the end of each test series. Cultivation broth samples (100 μL) were systematically drawn from all test series to test strain purity in agar plates incubated under CH_4:O_2 atmosphere (35:65 % *v/v*) in 2 L-Tedlar bags.

2.3 Analytical methods

CH_4, O_2, CO_2 and H_2S gas concentrations were determined according to López et al. in a Bruker 430 GC-TCD (Bruker, Palo Alto, USA) equipped with a CP-Molsieve 5A (15 m × 0.53 mm × 15 mm) and a CP-PoraBOND Q (25 m × 0.53 mm × 10 mm) columns [21]. The determination of OD_{600} and TSS concentration was performed as described elsewhere [22]. SO_4^{2-} concentration in the liquid phase was determined by HPLC-IC according to López et al. [21]. Cultivation broth samples of 1 mL were filtered (0.22 μm) and acidified with 20 μL H_2SO_4 (96-97 % (*w/v*)) prior to VFAs analysis in an

Agilent 7820A GC-FID (Agilent Technologies, Santa Clara, USA) equipped with a G4513A autosampler and a Chromosorb WAW packed column (2 m × 1/8" × 2.1 mm SS) (10 % SP 1000, 1 % H_3PO_4, WAW 100/120) (Teknokroma, Barcelona, Spain). The injector, oven and detector temperatures were maintained at 375, 130 and 350 °C, respectively. N_2 was used as the carrier gas at 45 mL min^{-1}.

Cultivation broth samples of 1.5 mL were centrifuged for 5 min at 13000 rpm and further processed according to López et al. [22]. The PHAs extracted from the samples were measured in a 7820A GC coupled with a 5977E MSD (Agilent Technologies, Santa Clara, USA) and equipped with a DB-wax column (30 m × 250 µm × 0.25 µm). The detector and injector temperatures were maintained at 250 ºC. The oven temperature was initially maintained at 40 ºC for 5 min, increased at 10 ºC min^{-1} up to 200 ºC and maintained at this temperature for 2 min. Finally, the oven temperature was increased up to 240 ºC at a rate of 5º C min^{-1}. The PHA content (wt %, w_{PHA}/w_{TSS}) of the samples, and the HB and HV fractions of the PHAs (mol %) were refered to the initial biomass concentration of the sample. The time course of the PHA yield (Y_{PHA}), based on the consumption of CH_4 or CH_4 and VFAs (and therefore expressed as gPHA gCH_4^{-1} or gPHA $gSubstrate^{-1}$, respectively), was estimated by dividing the mass of PHA produced by the mass of the substrate consumed at each sampling interval. The stoichiometry, kinetics and carbon distribution calculations were described in the Supporting Information section.

2.4 Statistical analyses

Arithmetic mean values and standard deviations were calculated for the replicate bottle cultures. The statistical data analysis was performed using OriginPro 8.5 (OriginLab Corporation, USA). The occurrence of significant differences within the data sets was analysed by a one-way analysis of variance (ANOVA) and a Tukey test. A Levene test

was also applied to study homocedasticity. Differences were considered significant at $P \leq 0.05$.

3. Results and discussion

3.1 Biogas-based growth and PHA synthesis by *M. hirsuta*

Abiotic controls showed neither a significant CH_4/O_2 consumption nor CO_2 production along Test Series 1 and 2 (Figure S1, S2). On the other hand, the presence of CO_2 and H_2S (artificial biogas with/without H_2S) did not result in significant differences either on *M. hirsuta* growth or CH_4/O_2 consumption compared to the cultures provided exclusively with CH_4 under N-sufficient conditions in Test Series 1 (Figure 2A, B, C; Figure S2A). Thus, CH_4 at 204 ± 3 g m^{-3} was completely depleted within 12 days of cultivation, which entailed the concomitant consumption of 78 % of the O_2 initially supplied, regardless of the headspace composition. Despite the fact that the presence of CO_2 could theoretically increase biomass productivity in methanotrophic cultures, similar net CO_2 and TSS productions of 279 ± 5 g m^{-3} and 619 ± 16 mg L^{-1}, respectively, were recorded regardless of the cultivation headspace (Figure 2, Figure S3A) [15]. In this context, the fraction of electrons used for energy generation (f_e) and cell assimilation (f_s) (estimated according to Rostkowski et al. [23], Supporting Information), the specific growth rates (μ) and the biomass yields (Y_X) were 0.48 ± 0.03, 0.52 ± 0.03, 0.30 ± 0.03 d^{-1} and 0.58 ± 0.03 gTSS gCH_4^{-1} under the three headspace compositions evaluated (Table S1). These values, which are strain-dependent and greatly influenced by the cultivation conditions, were identical to those previously reported for *M. parvus* OBBP when nitrate was used as nitrogen source [23]. These findings reinforced the hypothesis that neither CO_2 nor H_2S negatively affect the metabolism of type II species despite the decrease in the initial pH down to 6.12 ± 0.04. Surprisingly, H_2S rapidly disappeared from the headspace without resulting in an

increase in SO_4^{2-} concentration in the culture broth, which suggested that it was completely dissolved into the liquid phase (data not shown). It must be noted that other biodegradable volatile sulfur compounds (i. e. COS or CS_2) can be present in a real biogas and, therefore, slightly modify the pH and SO_4^{2-} concentration in the culture broth, though further assessments are required to confirm their effect on the growth of type II methanotrophs such as *M. hirsuta* [24]. In addition, no culture contamination by heterotrophic bacteria was found during periodic microscopy analysis and the culture streak plating performed in Test Series 1 (and along Test Series 2, 3 and 4) (Figure S4).

Likewise, similar patterns of CH_4/O_2 consumption and biomass/PHA synthesis were observed during *M. hirsuta* cultivation in NFMS regardless of the headspace composition (Figure 2D, E, F, S3B). In this regard, the substrate partitioning parameter f_s increased up to 0.69 ± 0.02 compared to the previous growth phase as a result of the electron deviation to PHB accumulation, regardless of the headspace composition (Table S2). Accordingly, a 2-fold decrease in μ (0.14 ± 0.01 d^{-1}) was observed during the accumulation phase likely mediated by extra energy requirements derived from PHB synthesis and by the metabolic unbalances caused by the lack of N in the culture medium (Table S2). More than 80 % of the final content of PHA (identified as PHB) accumulated during the first 48–72 h of cultivation. The PHA content remained constant at 45 ± 1 % (corresponding to a maximum Y_{PHA} = 0.44 ± 0.03 gPHB gCH_4^{-1}) from day 5 onwards for the three headspace conditions evaluated, which confirmed the potential of artificial biogas (with and without H_2S) as a feedstock for biopolymer production (Figure 2D, E, F). The contents and yields of PHB here obtained were in agreement with previous findings on PHA production by *Methylocystis* species (PHA contents of 28–51 % and yields of 0.38–0.55 gPHB gCH_4^{-1}) [10,13-19,25,26]. It should be stressed

that the carbon mass balances carried out for Test Series 1 and 2 entailed an error < 10 %, which validated both the analytical and instrumental methods used in the current study (Tables S1, S2). Finally, the results here obtained suggest the possibility to grow and coproduce PHA with other type II methanotrophic species belonging to *Methylocystis*, *Methylosinus*, *Methylocella* and *Methylocapsa* genera, since the presence of CO_2 and H_2S tend to decrease the pH within the culture broth and type II methanotrophs tolerate low values of pH (down to 4-5) [26].

<Fig. 2>

3.2 Effect of VFAs concentration on biogas-based *M. hirsuta* growth

Cosubstrate-deprived controls consumed CH_4 within the first 48–72 h, exhibiting similar CH_4 consumption rates to those obtained for acetic and propionic-supplemented cultures at C1 (Figure 3A, B; Figure S5). Acetic and propionic acids exhibited the largest inhibition on CH_4 consumption and *M. hirsuta* growth, C2 representing the highest concentration tolerated by this methanotrophic strain (Figure 3A, B; Table S3). In fact, negligible CH_4 and VFA degradations were observed at acetic and propionic acid concentrations higher than C3 (Figure 3A, B; Figure S6A, B). It must be also highlighted that the higher the concentration of acetic and propionic acids consumed by the strain, the higher the final TSS concentration reached (Table S3). Unlike type I methanotrophs, type II methanotrophs can grow at low pHs (4–7) and possess a complete tricarboxylic acid cycle pathway, including the α-ketoglutarate dehydrogenase enzyme that enables growth on organic acids [11,27]. However, the tolerance of type II methanotrophs to acetic acid seems to be species-dependent. For instance, repression of the transcription of the methane monooxygenase enzyme at acetate concentrations as low as 30 mg L^{-1} has been reported for *Methylocella* spp., while *Methylocystis* spp. and *Methylocella silvestris* BL2 can grow at acetate concentrations of 210–360 mg L^{-1} [27-

29]. In our particular study, *M. hirsuta* tolerated and was able to grow below ~200 mg acetic acid L^{-1} and at lower pHs (4.21) than previous studies (Figure S6A; Table S3). On the other hand, propionic acid induced a more severe inhibition on CH_4 consumption in *M. hirsuta*, with a ~3-fold reduction in the volumetric CH_4 removal rates at ~125 mg L^{-1} (compared to the test conducted at 200 mg acetic acid L^{-1}) despite the higher pH values here encountered (5.13) (Figure 3B, Figure S6B; Table S3). Similarly, Wieczorek et al. [30] reported the occurrence of a simultaneous biodegradation of CH_4 and propionate at ~180 mg L^{-1} and pH 4.8–5.1 in *Methylocystis* sp. cultures. Previous studies observed toxic effects of both acetate and propionate in *Methylocystis* species (pK_a values of 4.8 and 4.9, respectively) at pH < 4.8, where the protonated forms of these acids may alter the membrane potentials and uncouple ATP synthesis [30,31]. These findings are in agreement with the observed inability of our strain to grow at C3–C5 concentrations of acetic and propionic acids, and confirmed the hypothesis of a combined detrimental effect of pH and VFA on *M. hirsuta* growth.

On the contrary, the presence of butyric and valeric acids resulted in a lower detrimental effect on the ability of *M. hirsuta* to degrade CH_4, which can be attributed to the higher pHs induced by these VFAs in the cultivation medium (4.52 > pH < 5.78) (Figure 3C, D; Figure S6C, D; Table S3). Similar volumetric CH_4 consumption rates to those in cosubstrate-deprived controls were found in butyric-supplemented cultures at C1, though 2.7 times lower rates were obtained in the valeric-supplemented cultivations at the lowest concentration here tested (Figure 3C, D; Figure S5). Hence, *M. hirsuta* tolerated up to 273 ± 2 mg butyric acid L^{-1} (C4) and 177 ± 3 mg valeric acid L^{-1} (C3). In this context, the higher final TSS concentrations were recorded at increasing VFA concentrations consumed (Table S3). The maximum concentrations of butyric and valeric acids here tolerated by *M. hirsuta* were in the range of VFA concentrations

previously tested in CH_4-based PHA production studies under controlled pH conditions (100–120 mg butyrate L^{-1} and 100–400 mg valerate L^{-1}) [16-18].

Finally, biogas-deprived controls cultivated at C2 concentration for each VFA suggested the ability of *M. hirsuta* to grow on acetic, propionic, butyric and valeric acids under nutrient-sufficient conditions (Figure S7). Surprisingly, the VFA consumption rates were slightly lower compared to those obtained when artificial biogas was also supplied, which could be due to a higher activity of the C-1 driven electron transport chain enhanced by the oxidation of CH_4 (Figure S6). To the best of the authors' knowledge, this is the first work postulating the growth of a type II methanotroph on butyrate, valerate and propionate as the sole carbon and energy source. However, further experiments (i. e. based on isotope labeling) should be carried out to confirm the ability and consumption kinetics of these VFAs by *M. hirsuta*. In addition, since the ability to grow on individual volatile fatty acids with or without biogas seems to be species-dependent, further assessments in other type II methanotrophic strains are required prior implementation in methanotroph-based biorefineries.

<Fig. 3>

3.3 Evaluation of the tailored PHA production via integration of artificial biogas and VFAs biodegradation

C2 concentration was thus selected for the integration of both artificial biogas and VFAs biodegradation towards a tailored PHA synthesis during Test Series 4. No significant CH_4 or VFA degradation was recorded in the abiotic controls, which ruled out any potential removal of these compounds due to adsorption or photolysis (Figures S8, S9). The supplementation of the cultivation broth with acetic, butyric and propionic acids in the absence of biogas under N limitation did not enhance PHA accumulation,

the maximum PHA content being 2.8 % (> 91 % mol 3HB fraction) (Table 1). Surprisingly, the highest content of PHAs among the control tests without biogas was obtained with valeric acid (up to 9.0 ± 1.7 % PHA), which entailed a high 3HV fraction of 83 mol %. These results suggested that the synthesis of PHV in *M. hirsuta* was not strictly linked to the assimilation of CH_4. In contrast, Myung et al. [17] found that *Methylocystis parvus* OBBP was not able to accumulate PHAs using valerate as the sole carbon and energy source, which highlights the higher metabolic versatility of *M. hirsuta*. It must be noted that no biomass formation was observed during *M. hirsuta* cultivation on VFAs as the sole carbon source during the accumulation phase, where the carbon belonging to VFAs was mainly deviated towards the production of CO_2 (Table S4). The use of artificial biogas as the sole substrate during the accumulation phase supported similar maximum Y_{PHA} and PHA contents to those found during Test Series 2 (0.41 ± 0.02 gPHA gSubstrate^{-1} and 43 ± 2 %, respectively), with 3HB as the main monomer within the biocomposite (Table 1, Table S4).

<Table 1>

The addition of propionic, acetic or butyric acid as co-substrates during biogas biodegradation by *M. hirsuta* enhanced PHA accumulation, which increased from 43.1 ± 1.8 % up to 47.9 ± 0.7, 52.3 ± 0.7 and 52.2 ± 2.1 %, respectively. This represented a 10–20 % increase in PHA accumulation over the basal content obtained only with artificial biogas and matched the increase in the maximum Y_{PHA} (by 10–30 %) achieved (Table S4). To the best of the authors' knowledge, there are no previous studies evaluating the supplementation of acetate as co-substrate during CH_4-based PHA accumulation by methanotrophic bacteria, which could presumably act as direct precursor for the synthesis of 3-hydroxybutyryl-CoA and thus, of 3HB units (Figure 4). The final PHA content here reported for the simultaneous cultivation of *M. hirsuta* in

propionic and biogas were higher than those found by Myung et al. using pure CH_4 and propionate at 100 mg L^{-1} (32 ± 4 %) [17]. Surprisingly, the 3HV fraction obtained by these authors with *M. parvus* OBBP (25 mol %) significantly differed from the one obtained in *M. hirsuta* in the present study (2 mol %), which suggests that propionic acid bioconversion in methanotrophic bacteria is species-dependent [17]. The PHA contents and HB:HV ratios here obtained were comparable to those obtained in *M. parvus* OBBP when butyrate was supplemented together with CH_4 (55 ± 3 %, 100:0 ratio) [18]. In our particular study, the highest PHA content was found when valeric acid was used as co-substrate during biogas-based *M. hirsuta* cultivation, which resulted in a final PHA content of 53.8 ± 0.8 % (corresponding to a maximum Y_{PHA} = 0.63 ± 0.05 gPHA $gSubstrate^{-1}$) and a 3HB:3HV ratio of 75:25 (Table 1, Table S4). Maximum PHA contents of 54 ± 4 %, with 3HB:3HV ratios of 75:25 and Y_{PHA} of 0.67 gPHA $gSubstrate^{-1}$ have been reported in *Methylocystis* species when valerate was added together with CH_4 [16,18,19]. Likewise, previous studies have consistently demonstrated that fatty acids assimilation by methanotrophic bacteria is an energy intensive process, which increases f_e when co-substrates such as valerate are supplemented [17,32]. In this regard, Bahr et al. demonstrated that the lower the carbon oxidation-reduction state (CORS) of a pollutant, the higher the energy requirements (and therefore the oxygen demands) during the cultivation of a methanotrophic bacterial consortium [33]. In our particular study, a gradual increase in f_e was observed as the CORS of the VFA decreased. Thus, f_e increased from 0.52 ± 0.02 under acetic acid (CORS = 0) cultivation to 0.94 ± 0.05 under valeric acid (CORS = -6) co-addition, the later likely explaining the reduced biomass growth observed (Table S4). These f_e values were higher than those previously obtained during PHA accumulation under valerate

co-supplementation, which could be attributed to the lower pHs here encountered [16,17].

<Fig. 4>

4 Conclusions

Artificial biogas (with and without H_2S) supported a similar growth and PHA accumulation (under nitrogen limitation) to pure CH_4 in *M. hirsuta* cultures, which confirmed for the first time the feasibility of biogas-based biorefineries devoted to the production of these high-added value product. *M. hirsuta* was able to use acetic, butyric, propionic and valeric acids as the sole carbon and energy source. This study also demonstrated the potential of the individual supplementation of these VFAs to modify the composition of the biocomposite, valeric acid supporting up to 25 % HV fraction within the whole biopolymer. Further research is still needed to elucidate the effect of i) including other trace gases within the biogas (i. e. volatile sulfur compounds such as COS or CS_2), and ii) the multiple supplementation of VFAs on biogas-based *M. hirsuta* growth and PHA synthesis.

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Table 1. Final content and composition of the PHA synthesized by *M. hirsuta* during the biodegradation of biogas and/or VFAs under N limitation (Test Series 4).

Culture condition	PHA		
	PHA content (% w_{PHA}/w_{TSS})	**3HB fraction (mol %)**	**3HV fraction (mol %)**
Biogas	43.1 ± 1.8	100	0
Acetic acid	2.4 ± 0.4	98	2
Propionic acid	1.1 ± 0.7	91	9
Butyric acid	1.8 ± 0.9	99	1
Valeric acid	9.0 ± 1.7	17	83
Biogas + Acetic acid	52.3 ± 0.7	100	0
Biogas + Propionic acid	47.9 ± 0.7	98	2
Biogas + Butyric acid	52.2 ± 2.1	100	0
Biogas + Valeric acid	53.8 ± 0.8	75	25

Figure 1

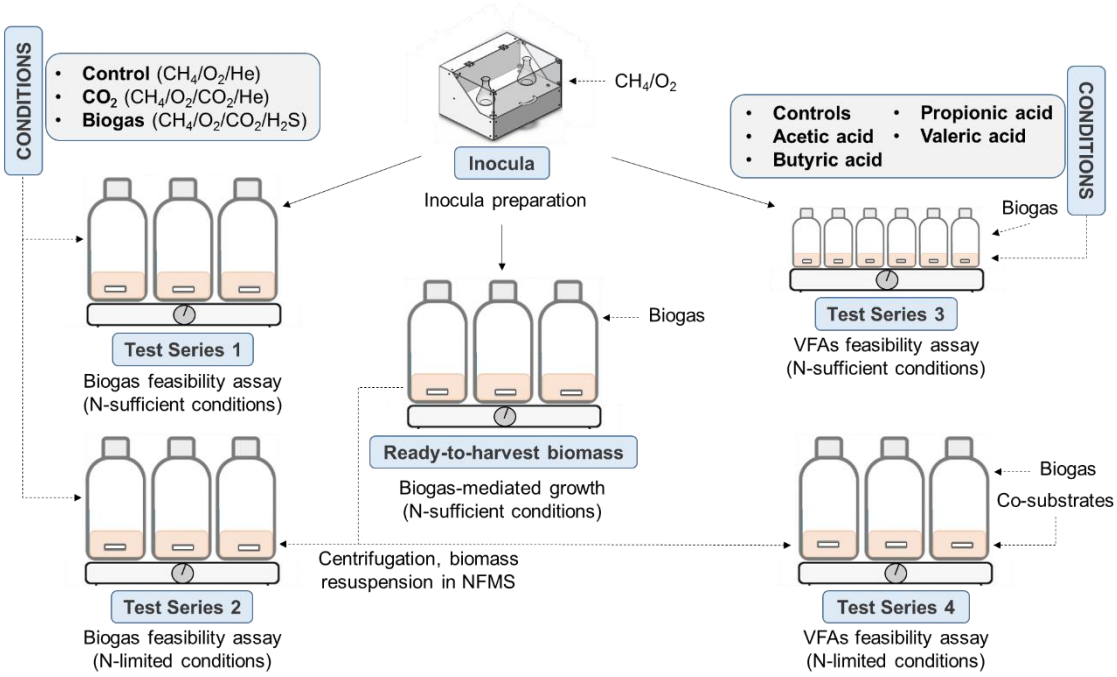

Figure 1. Test series overview.

Figure 2

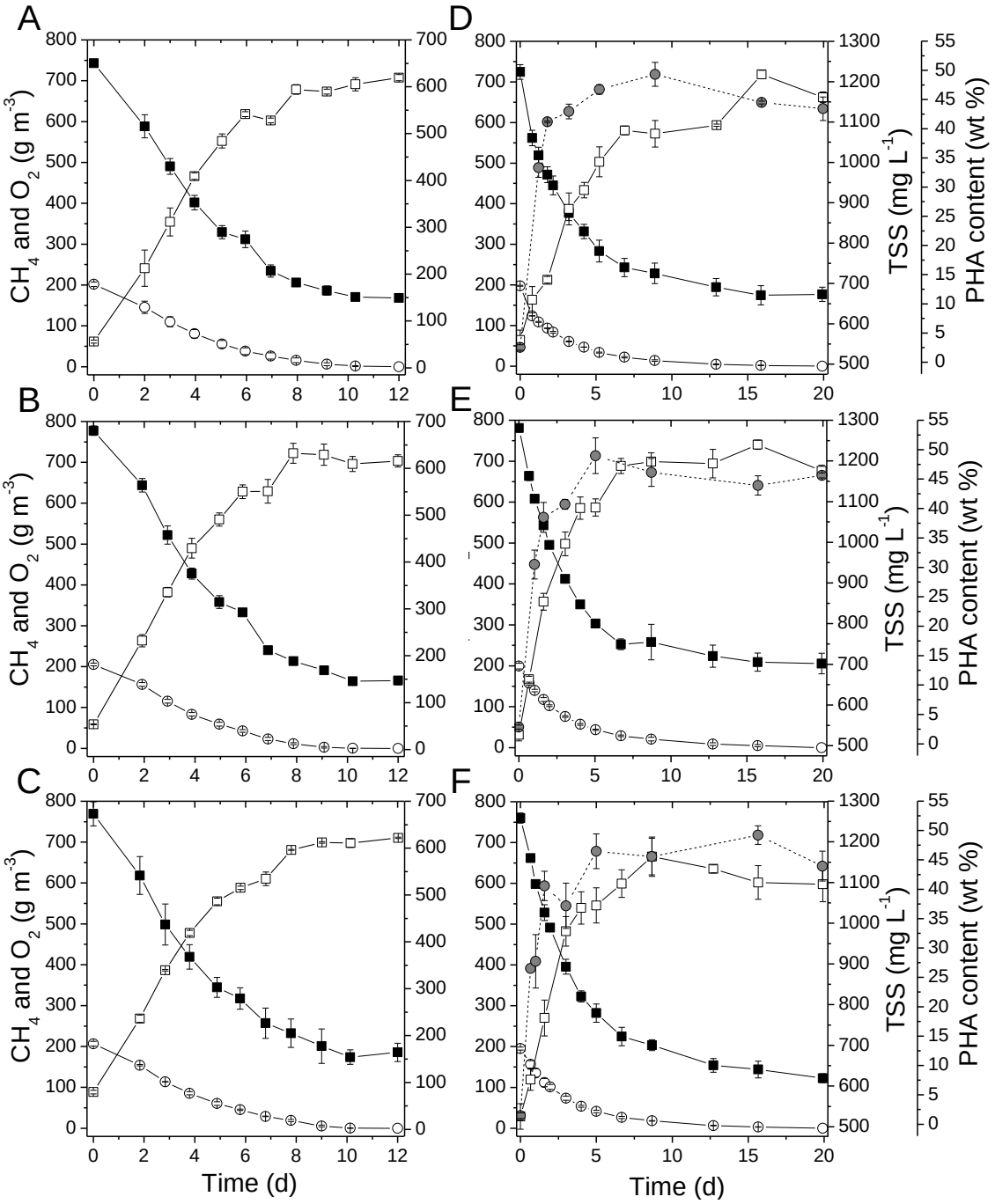

Figure 2. Time course of CH_4 (○), O_2 (■), TSS (□) and PHA (●) concentrations during the growth phase of Test Series 1 (A–C) and during the accumulation phase of Test Series 2 (D-F) using CH_4 (A, D), H_2S-free biogas (B, E) and biogas (C, F) as carbon and energy source. PHA content was not represented during the growth phase since contents below 1 % were found regardless of the headspace composition.

Figure 3

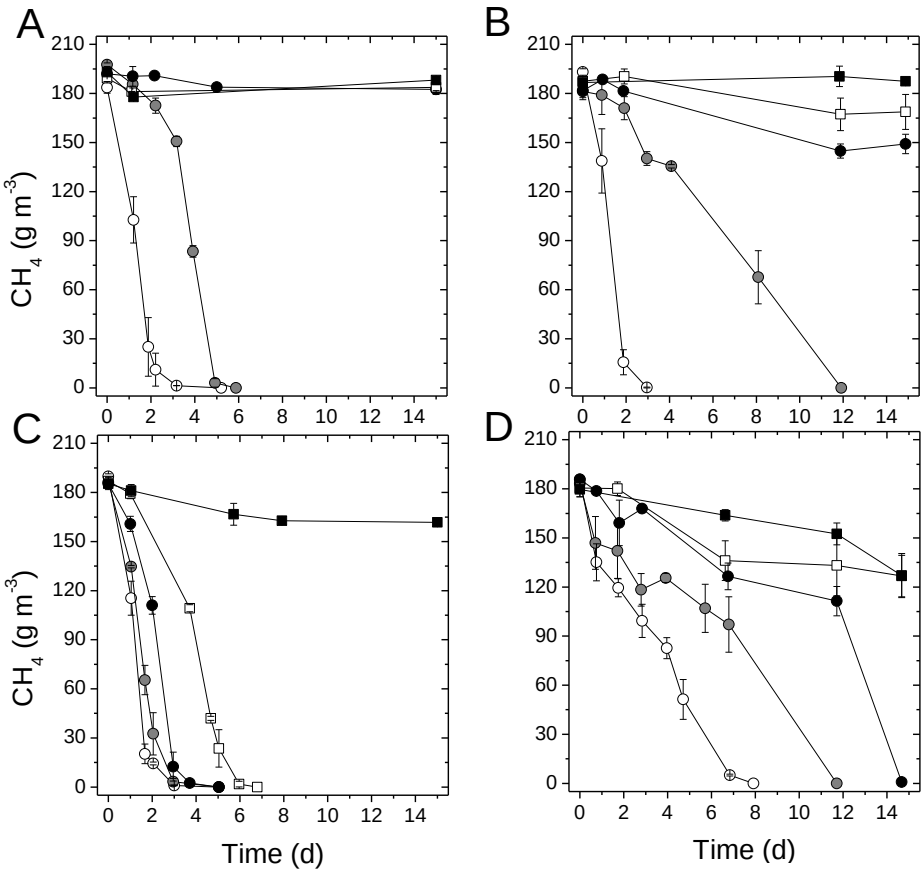

Figure 3. Time course of CH_4 concentration in the presence of A) acetic, B) propionic, C) butyric and D) valeric acids at C1 (○), C2 (◍), C3 (●), C4 (□) and C5 (■) concentrations (Test Series 3).

Figure 4

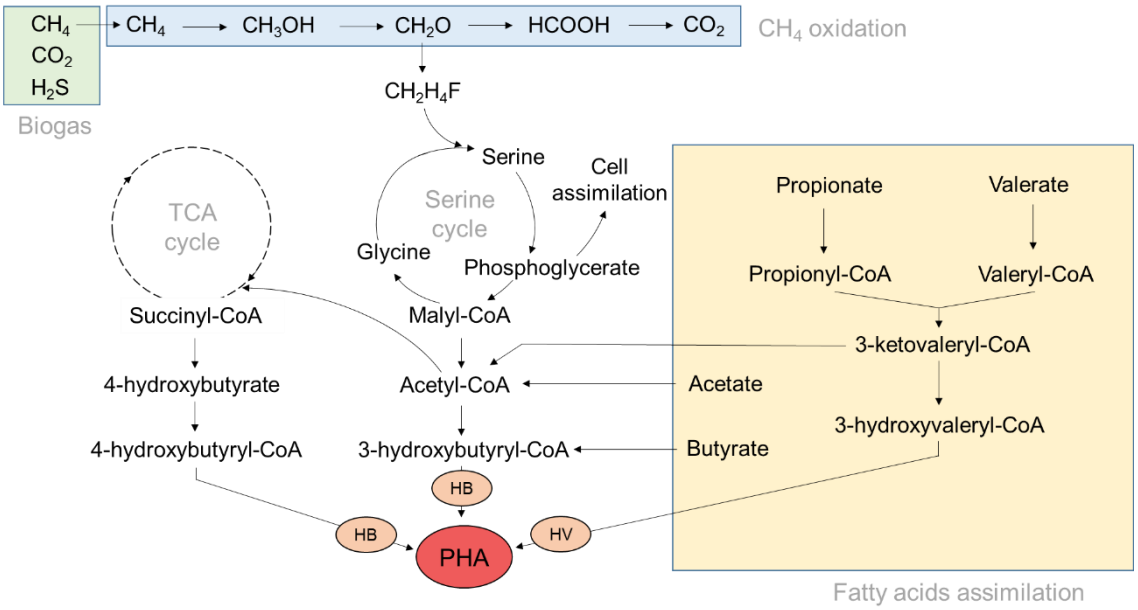

Figure 4. Tentative PHA production pathway for type II methanotrophs using both biogas and VFAs as carbon sources. Dotted arrows indicate the existence of intermediates not mentioned for clarity purposes. The pathways proposed are based on previous CH_4-driven PHA accumulation studies [16,18].

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