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The co-conversion of methane and mixtures of volatile fatty acids into poly (3-hydroxybutyrate-co-3-hydroxyvalerate) expands the potential of an integrated biorefinery

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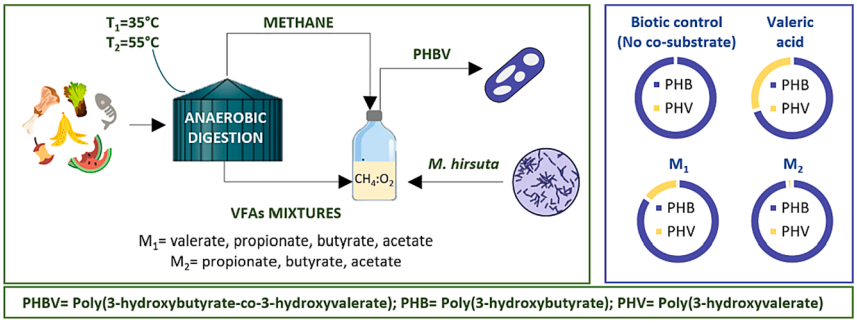
HIGHLIGHTS

- No 3-HV monomers accumulated with methane as the sole carbon source.
- Methane and valeric acid enabled the accumulation of PHBV.
- VFA streams containing valerate and propionate supported PHBV synthesis.
- VFA mixture composition impacted the molar structure of the polymer produced.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work, the potential of *Methylocystis hirsuta* to simultaneously use methane and volatile fatty acids mixtures for triggering PHBV accumulation was assessed for the first time batchwise. Biotic controls carried out with CH_4 alone confirmed the inability of *Methylocystis hirsuta* to produce PHBV and achieved 71.2 ± 7 g $m^{-3}d^{-1}$ of PHB. Pure valeric acid and two synthetic mixtures simulating VFAs effluents from the anaerobic digestion of food waste at 35 °C (M_1) and 55 °C (M_2) were supplied to promote 3-HV inclusion. Results showed that pure valeric acid supported the highest polymer yields of 105.8 ± 9 g $m^{-3}d^{-1}$ (3-HB:3-HV=70:30). M_1 mixtures led to a maximum of 103 ± 4 g $m^{-3}d^{-1}$ of PHBV (3-HB:3-HV=85:15), while M_2 mixtures, which did not include valeric acid, showed no PHV synthesis. This suggested that the synthesis of PHBV from VFAs effluents depends on the composition of the mixtures, which can be tuned during the anaerobic digestion process.

1. Introduction

Nowadays, plastic pollution is a global concern that seriously threatens human health and the environment (Horton, 2022; Iroegbu et al., 2021). On European beaches, plastics account for 80–85 % of marine litter and cost to the European Union economy an estimated 259–695 million € per year (Halleux, 2019). A study published in 2015 revealed that a staggering 4.8 to 12.7 million tonnes of plastic find their

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way into the oceans each year (Jambeck et al., 2015). Much of this plastic debris into the ocean is out of sight since waste has also been found at depths of 6000 m (Chiba et al., 2018). Moreover, the very wide range of plastics size, which can be classified as macro, micro and nanoplastics, makes its significant impact at different levels (Iroegbu et al., 2021). For example, many animals in the marine environment have been found trapped in six-ring plastic or strangled by other fishing items. Also, the tiniest organisms (i.e. plankton) can be poisoned by ingestion of nanoplastics and, since many animals depend on it for food, a negative impact is observed on the entire food chain (Cole et al., 2013; Setälä et al., 2014). The presence of harmful additives, such as plasticizers, UV stabilizers and pigments, also entails a correlation between exposure to plastics and a multitude of health problems affecting humans worldwide (Gallo et al., 2018; Thompson et al., 2009). For example, the ingestion or inhalation of micro and nanoplastics has been related to microbiota alterations, mutagenic effects on DNA, oxidative stress and neurotoxicity among the others (Iroegbu et al., 2021).

Given the problems mentioned above, there is a heightened focus on finding a sustainable alternative to conventional plastics (Horton, 2022; Iroegbu et al., 2021). In this context, bioplastics have emerged as the most promising alternative to fossil-based polymers and encompass plastic materials that are either bio-based (i.e. wholly or partly derived from biomass), biodegradable (i.e. can be broken down by microorganisms like fungi or bacteria into water, CO_2 and biomass) or feature both characteristics (Moshood et al., 2022; Rosenboom et al., 2022; Verma and Fortunati, 2019).

Polyhydroxyalkanoates (PHAs) are promising biopolymers for potentially replacing fossil-based plastics, primarily due to their unique properties (Acharjee et al., 2023; Pittmann and Steinmetz, 2017). PHAs are produced by several gram-positive and gram-negative bacteria under nutrient limitation and can result from the combination of more than 150 monomers (Chee et al., 2010; Reddy et al., 2003). In this context, novel species that could increase PHAs yields are continuously discovered. Thus, Lee et al. (2022) recently reported that a novel *Loktanella* sp. SM43 was able to accumulate up to 66.5 % w w^{-1} of PHB using glucose as the main carbon source (S. M. Lee et al., 2022b). Likewise, a new species of Bacillus sp. YHY22 yielded up to ≈ 65 % w w^{-1} of PHB using lactate (H. J. Lee et al., 2022), while *Halomonas sp*. YK44 was reported to produce up to 78 % w w^{-1} of PHB from galactose (Jung et al., 2022).

The different types and length of monomers forming PHAs affect their biodegradability, hydrophobicity, melting point, Young's modulus, water vapour and oxygen transmission rate and glass transition temperature (Wolf et al., 2005). For example, PHB homopolymer has a higher crystallinity and molecular mass than co-polymers such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBH) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate) (PHBVH) (Lee et al., 2021). The combination of 3-hydroxybutyrate (3-HB) and 3-hydroxyvalerate (3-HV) emerges as highly appealing since the resulting polymer, known as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), exhibits similar properties to widely utilized conventional plastics. For instance, its mechanical and thermal properties are comparable to those of polypropylene, with the benefits of being biodegradable, non-toxic and biocompatible (Policastro et al., 2021). Moreover, PHBV is suitable for a wide range of applications, such as packaging, medical devices or for combination with other polymers, carbon nanomaterials and fibres (Lin et al., 2021).

The properties of PHBV vary depending on its molecular structure, i.e. the ratio between the fractions of 3-HB and 3-HV (Cal et al., 2016), which in turn relies on the substrates used during microbial cultivation. Indeed, the inclusion of 3-HV monomers reduces the melting temperature and the glass transition temperature of the polymer, its crystallinity and water permeability in an extent that depends on the fraction of 3-HV (Amabile et al., 2023; Policastro et al., 2021).

Many feedstocks (i.e. crude glycerol, glucose, sugarcane molasses, methanol, detoxified hydrolysates and starch) have been tested to date for their bioconversion into PHAs, some of them being cheap, renewable, and easy to find (Bhatia et al., 2023; Kim et al., 2023; Yamane, 1993). Methane, for instance, is a high available and cheap substrate that is metabolized by several type II methanotrophs for its conversion into poly(3-hydroxybutyrate) (PHB), but it cannot be used alone to foster the biosynthesis of 3-HV building blocks (Policastro et al., 2021). For this purpose, several strategies to produce PHBV have been explored in the past years based on the selection of the most appropriate feedstock, the choice of the bacterial strain or the use of engineered co-cultures (Bhatia et al., 2018; Policastro et al., 2021). In the particular case of methane-based PHBV production, the use of specific precursors for 3-HV inclusion, such as volatile fatty acids (VFAs), appears as the most suitable strategy. For instance, Lopez et al. (2018) investigated the effects of propionic, valeric, butyric and acetic acids during their co-conversion with methane by *Methylocystis hirsuta* into PHBV individually. The authors found that the highest 3-HV content was obtained when using valeric acid at low concentrations (≈90 ppm), while higher concentrations of the precursor (>200 ppm) inhibited microbial activity, probably because they induced a severe reduction of the cultivation pH (López et al., 2018). Myung et al., 2015 also reported that the conversion of methane coupled with 100 and 400 ppm of valeric acid in a mixed methanotrophic consortium resulted in PHBV contents of 43 % w w^{-1} (20 % mol 3-HV) and 30 % w w^{-1} (40 % mol 3-HV), respectively (Myung et al., 2015).

Unfortunately, despite the successful production of PHBV with different molecular structures, using costly pure acids could challenge the economic viability of such processes at the industrial scale (Lin et al., 2021). Indeed, the high production costs related to PHAs production, which are mainly caused by the cost of the feedstock and the downstream process, could be further increased by the acquisition of pure VFAs (Kumar et al., 2020; Wang et al., 2022). In this context, using VFAs from the anaerobic digestion of organic waste, could represent a viable opportunity to improve PHBV production and reduce the overall process costs (Al Battashi et al., 2021; Jiang et al., 2013). Moreover, the composition of the VFAs mixture can also be tuned by varying the operating conditions during anaerobic digestion (i.e. temperature, pH and organic load rate) to provide the ideal precursors for PHBV biosynthesis during methane bioconversion (Jiang et al., 2013).

This work aimed at assessing the potential of an integrated biorefinery concept in which methane and mixtures of VFAs, which can both be produced during the anaerobic digestion of the organic waste, are converted by a methanotrophic platform into the biodegradable PHBV. First, methane and valeric acid at two different concentrations were supplied to confirm the ability of *Methylocystis hirsuta*, which was previously reported among the best performing methanotrophs, to accumulate PHBV (García-Pérez et al., 2018a; Rodríguez et al., 2020). Then, the potential of two VFAs mixtures to support PHBV synthesis in *Methylocystis hirsuta* cultures supplied with methane was investigated. The effects of the mixture composition on the polymer yields and on its molecular structure were analysed in order to investigate the feasibility of using VFAs streams from the anaerobic digestion of food waste for producing high quality PHAs from methane as the main carbon source. It is worth highlighting that, despite VFAs mixtures were previously investigated to produce PHBV, there are no such studies, to the authors' knowledge, performed with methanotrophs using methane as the main carbon source and VFAs mixtures as co-substrates.

2. Materials and methods

2.1. Chemicals

The chemicals used for the preparation of the culture medium were acquired from PANREAC AppliChem (Barcelona, Spain) except for $CoCl_2$, FeEDTA, $Cl_2Ni{\cdot}6H_2O$, $ZnSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$, H_3BO_3, $NiCl_2{\cdot}6H_2O$, $Na_2HPO_4{\cdot}12H_2O$, $Na_2MoO_4{\cdot}2H_2O$, which were obtained

from Sigma Aldrich. KNO_3 was purchased from Labkem (Barcelona. Spain). Valeric (≥99%), propionic (≥99%), butyric (≥99%) and acetic acid (99%) were acquired from Sigma Aldrich. Chloroform (≥99.8%), 1-propanol (99.7%), benzoic acid (99.5%), hydrochloric acid (37 % w v^{-1}) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV, 12 % mol 3-HV) (99.99%) were obtained from Sigma Aldrich and used for extracting and measuring PHBV. Oxygen (99.5%) and methane (99.5%) were provided by Abelló Linde S.A. (Spain) and Carburos Metalicos (Spain), respectively.

2.2. Strains, culture medium and VFAs mixtures

The methanotrophic PHA producer used in this work was *Methylocystis hirsuta* CSC1, which was purchased from Leibniz-Institut (DSMZ, Germany) and selected based on its ability to accumulate high amounts of PHAs from methane and VFAs (López et al., 2018). The strain was grown in a mineral salt medium (NMS) containing the following macronutrients (g/L): 0.2 $CaCl_2{\cdot}2H_2O$, 1.0 KNO_3. Micronutrients were also supplied by adding 1 mL of the following trace element solution (mg L^{-1}): 0.38 Fe-EDTA, 0.4 $Na_2MoO_4{\cdot}2H_2O$, 0.3 $Na_2EDTA{\cdot}2H_2O$, 1.0 $CuSO_4{\cdot}5H_2O$, 0.5 $FeSO_4{\cdot}7H_2O$, 0.4 $ZnSO_4{\cdot}7H_2O$, 0.015 H_3BO_3 0.01, 0.03 $CoCl_2$, 0.02 $MnCl_2{\cdot}4H_2O$, $NiCl_2{\cdot}6H_2O$ The initial pH was adjusted to 6.8 by adding 10 mL of a buffer solution of 72 g L^{-1} $Na_2HPO_4{\cdot}12H_2O$ and 26 g L^{-1} KH_2PO_4. The same culture medium described above, deprived of KNO_3 and enriched with VFAs, was used to induce the microbial accumulation of PHBV. In this context, solutions of pure valerate and two different mixtures of VFAs, named M_1 (36.5% acetate, 31.7% propionate, 21.9% butyrate and 9.9% valerate) and M_2 (15.4% acetate, 3.5% propionate and 81.1% butyrate) were prepared and supplied in order to represent 15% and 30% of the total carbon (C_{tot}). More specifically, since the total carbon was the sum of the carbon in the gas and the liquid phase ($C_g + C_l$), the fraction of carbon to be added in the form of VFAs into the culture medium was directly determined. Two solutions (C_{VFA}: 15% and 30% of C_{tot}) of 20 mL each were prepared for valerate, M_1 and M_2 mixtures based on the compositions compiled in Table 1. Note that, in this study, the VFA mixtures were designed in order to simulate the VFA composition potentially extracted from the anaerobic digestion of food waste. In particular, a mixture with valeric acid (M_1) and a mixture without valeric acid (M_2), which correspond to two different operational conditions of the anaerobic digestion, i.e. mesophilic regime (35 °C) and thermophilic regime (55 °C), were investigated. In a real biorefinery context, VFAs must be first extracted from the digestate, since anaerobic digestates contain high concentrations of nitrogen, which is not suitable for PHAs production.

2.3. Experimental procedures

2.3.1. Inocula preparation and Methylocystis hirsuta cultivation

Methylocystis hirsuta CSC1 was first grown by transferring 5 mL of a stock culture to sterile 125 mL serum bottles containing 50 mL of MSM. Cultures were capped with butyl-rubber stoppers, crimp-sealed and placed in an orbital shaker at 200 rpm and 25 °C for a period of 6 days. The headspace was filled with a mixture of O_2:CH_4 (2:1 v v^{-1}), which was replenished every 48 h. The refreshment process involved flushing oxygen into the headspace for 5 min and then replacing 25 mL with methane using a 50 mL gastight syringe (Hamilton 1050 TLL, USA). It is worth highlighting that, under full scale application, the concentration of CH_4 in air must be lower than 5 % v v^{-1} to avoid explosion hazards, which will still support PHA accumulation under nitrogen deficiency (García-Pérez et al., 2018b). Once the cultures were active, sterile 2.15 L serum bottles containing 0.5 L of NMS were inoculated with 25 mL of *Methylocystis hirsuta* inoculum. The inoculation took place under a O_2:CH_4 atmosphere with a ratio of 2:1 v v^{-1}. The bottles were then agitated on multipoint stirrers (Variomag, Thermo Fisher Scientific, USA) at 300 rpm and 25 °C. The headspace composition, the optical density at 600 nm (OD_{600}) and the total suspended solid concentration (TSS) were measured at intervals of 48 h.

Table 1
Composition of valeric acid and VFAs mixture solutions used during PHBV accumulation.

Acids concentration for 20 mL of solution	VA 15 %C_{tot}	VA 30 %C_{tot}	M_1 15 %C_{tot}	M_1 30 %C_{tot}	M_2 15 %C_{tot}	M_2 30 %C_{tot}
Valeric acid [M]	0.035	0.084	0.004	0.01	–	–
Propionic acid [M]	–	–	0.02	0.047	0.002	0.005
Acetic acid [M]	–	–	0.028	0.067	0.011	0.026
Butyric acid [M]	–	–	0.011	0.027	0.038	0.093

2.3.2. PHBV accumulation by Methylocystis hirsuta in valeric acid, M_1 and M_2 enriched medium

To assess the potential of *Methylocystis hirsuta* to accumulate PHBV, biomass was harvested through centrifugation of the inoculum grown according to the procedure described above (4200 rpm,10 min) by using a Sorvall X PRO series Centrifuge (Thermo Scientific TM). The pellet was then resuspended in 2.15 L serum bottles containing 0.5 L of a nitrogen-free salt medium (NFSM) enriched with VFAs. After being crimp-sealed, the bottles were filled with a methane-oxygen atmosphere following the procedure described above and incubated at 350 rpm and 25 °C using multipoint stirrers. During this phase, three distinct conditions were examined through duplicate experiments. During the first test (i), valeric acid alone was supplied as 15% C_{tot} and 30% C_{tot} by adding 5 mL of valerate solutions (Table 1). In the second test series (ii), 5 mL of M_1 mixture were added to provide 15% and 30% of the total carbon. This solution was designed to replicate the composition of VFAs streams obtained from the anaerobic digestion of food waste at 35 °C (Jiang et al., 2013). In the third test series (iii), 15% C_{tot} and 30% C_{tot} were supplied through the addition of 5 mL of M_2 solution, which were formulated to match the VFAs composition obtained from anaerobic digestion of food waste at 55 °C (Jiang et al., 2013). Note that the solutions were prepared by diluting the acids with distilled water to the desired concentration and a total volume of 50 mL. The experimental setup is sketched in Fig. 1, while the composition of the solutions used is reported in Table 1. To avoid inhibitory effects, pH was adjusted to 7 during all test series. The atmosphere composition, OD_{600}, volatile fatty acid concentration, TSS and PHBV content were measured every 48h.

2.4. Analytical methods

The concentrations of methane, oxygen and carbon dioxide in the headspace of the bottles were measured every 48h using a Bruker 430 GC-TCD (Bruker Corporation, Palo Alto, USA) equipped with a CP Molsieve 5A and a CP-PoraBOND Q columns. Temperature was kept at 45, 150 and 200 °C in the oven, injector and detector, respectively. The OD_{600} was measured using a UV-2550 spectrophotometer (Shimadzu, Japan), while TSS was estimated according to 2540 APHA standard method (American Public Health Association, 2012). For VFAs measurements, samples were prepared by filtering 1 mL of culture broth (0.22 μm) and adding 30 μL H_2SO_4. Then, the collected samples were analysed by gas chromatography with a flame ionization detector using an Agilent 7820A GC-FID (Agilent Technologies, Santa Clara, USA). The temperature of the oven, injector and detector was kept at 130 °C, 375 °C and 350 °C, respectively. Nitrogen was used as carrier gas. Samples for PHAs extraction were prepared according to Rodríguez et al. (2020) and analysed via gas chromatography-mass spectrometry using a 7820A GC coupled with a 5977E MSD (Agilent Technologies, Santa Clara, USA) and equipped with a DB-wax column. Helium was used as carrier gas. The qualitative analysis of the polymer produced (PHB or PHV) and the quantification of the two monomeric units was carried out based on the results of GC measurements (López et al., 2018). The internal standard was benzoic acid, while PHB-co-12%HV was used as the external standard. Polymer accumulation as weight percentage of the

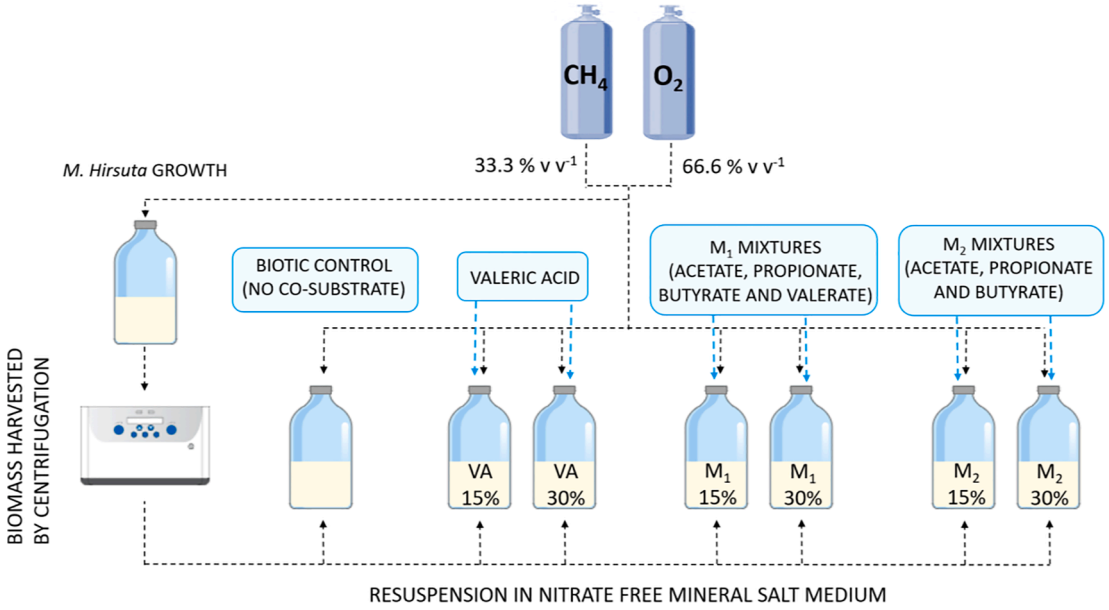

Fig. 1. Overview of the experimental setup during PHBV accumulation tests by *M. hirsuta* in valeric acid, M_1 and M_2 enriched medium.

total suspended solids was calculated on a dry basis.

3. Results and discussion

3.1. Growth of Methylocytis hirsuta and PHAs synthesis from CH_4

Biotic controls of *Methylocystis hirsuta* were grown on methane as the sole carbon and energy source and resuspended in a nitrate-free medium to assess the ability of the strain to produce PHB or PHBV in the absence of co-substrates. Biomass reached a concentration of 200 ± 0 mg TSS L^{-1} by day 7, which entailed the consumption of 85 ± 0.7 % v v^{-1} of the initial CH_4 and 46 ± 0 % v v^{-1} of the initial O_2 (Fig. 2a). The net CO_2 production accounted for 289.8 ± 6.5 g m^{-3}, which was very similar to the value reported by López et al. (2018) during the cultivation of *Methylocystis hirsuta* on biogas under a similar CH_4:O_2 ratio (López et al., 2018). Cells were harvested by centrifugation and resuspended on day 7. A maximum PHB content of 53.8 ± 9.9 % w w^{-1} was accumulated in *Methylocystis hirsuta* cultures by day 18, with 86% of the final PHB being produced during the first 48h after resuspension in N-free medium and accounting for a maximum productivity of 71.2 ± 7 g $m^{-3}d^{-1}$ (Fig. 2b). About 70.7 ± 2.1 % v v^{-1} of the replenished CH_4 and 52.5 ± 3.7 % v v^{-1} of the replenished O_2 were depleted by day 23 in the accumulation phase, with simultaneous production of 457.4 ± 38.7 g m^{-3} of CO_2. Similar results, in terms of PHAs yields and substrate consumption, were reported during the cultivation of *Methylocystis hirsuta* on methane under nitrogen-deprived conditions (López et al., 2018). No PHV fraction was produced when methane was fed as the sole carbon and energy source during the accumulation phase, the polymer composition being 100 % PHB (López et al., 2018; Myung et al., 2015). In this context, Luangthongkam et al. (2019) reported that a *Methylosinus*-dominated culture accumulated 8.6 % w w^{-1} of PHAs with 3-HB:3-HV ratio of 100:0 when using methane as the sole carbon and energy source (Luangthongkam et al., 2019). Likewise, Myung et al. (2016) also demonstrated that cultivating *Methylocystis parvus* OBBP in a co-substrates-deprived medium resulted in a 50 % w w^{-1} PHB accumulation but did not induce PHV accumulation (Myung et al., 2016). Indeed, while 3-hydroxybutyl CoA (3-HBCoA) can be derived from several substrates, including methane alone, 3-hydroxyvaleryl-CoA (3-HVCoA), which is responsible for the accumulation of 3-HV units, requires the addition of specific precursors, such as VFAs.

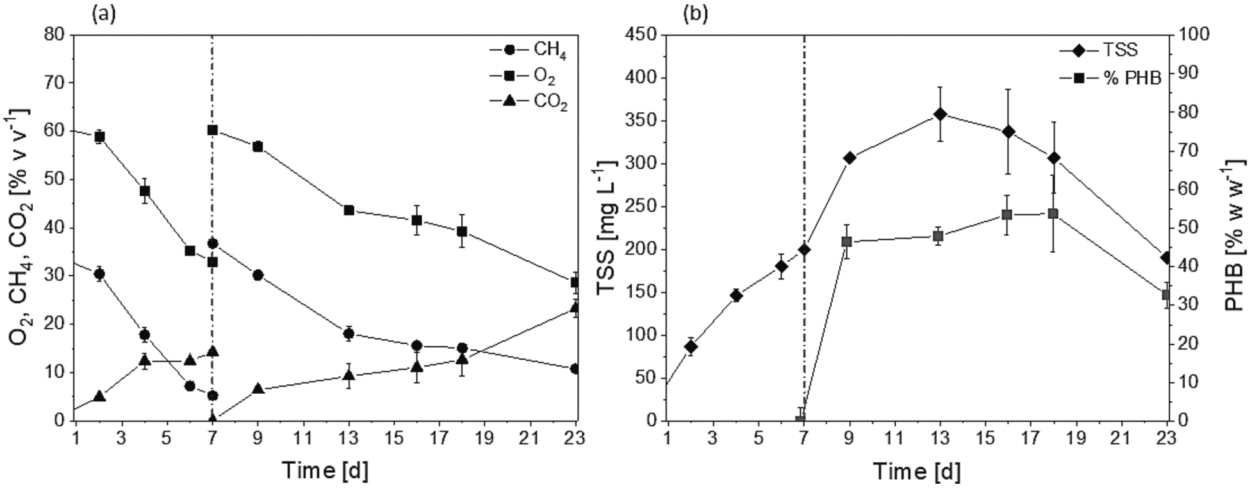

Fig. 2. Time course of methane, oxygen and CO_2 concentration (a) and TSS and PHB content in the absence of 3-HV precursors (b). The vertical dashed line by day 7 represents the beginning of *M. hirsuta* cultivation under nitrogen deprivation; PHB=*Poly(3-hydroxybutyrate)*.

3.2. PHBV synthesis by *Methylocystis hirsuta* from CH_4 in a valerate-supplemented medium

Valeric acid was selected as a co-substrate to assess the ability of *Methylocystis hirsuta* to accumulate PHBV in the presence of methane based on preliminary findings identifying valerate as the most promising precursor for 3-HV inclusion in PHA (Fergala et al., 2018). First, *Methylocystis hirsuta* was grown on methane and nitrate for 7 days, as previously described, up to concentrations of 185 ± 21 mg TSS L^{-1} and 200 ± 28 mg TSS L^{-1} and then resuspended in a nitrogen-deprived medium

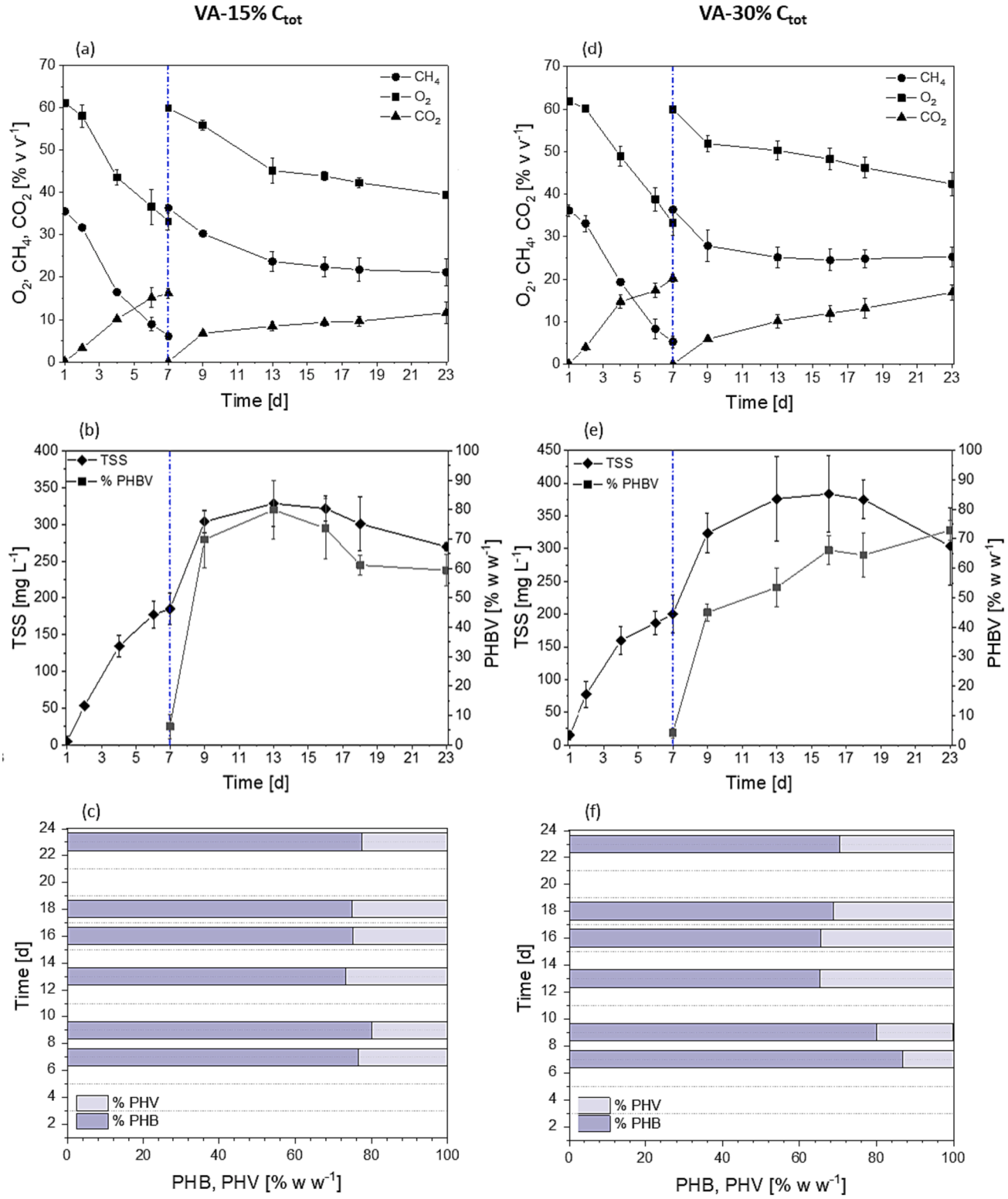

Fig. 3. Time course of methane, oxygen and carbon dioxide concentrations (a,d), TSS and PHBV concentrations (b,e) in *M. hirsuta* grown in a medium supplemented with valeric acid as 15%C_{tot} (a,b,c) and 30% of C_{tot} (d,e,f); PHBV=*Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)*; PHB=*Poly(3-hydroxybutyrate)*; PHV=*Poly(3-hydroxyvalerate)*.

enriched with valeric acid at 15 % (VA-15%) and 30 % (VA-30%) of the total carbon, respectively.

The addition of VA-15% (141 mg L^{-1}) mediated a PHBV content of 79 ± 0.7 % w w^{-1} by day 13, with a 3-HB:3-HV ratio of 73:27 (Fig. 3b and c), and a maximum PHBV productivity of 105.8 ± 9 g $m^{-3}d^{-1}$. These PHBV yields were higher than those previously reported for pure methanotrophs grown on methane and valeric acid (López et al., 2018; Myung et al., 2016). In this context, it should be noted that this study was designed to obtain a significant PHBV fraction using methane and VFAs as substrates instead of reaching a very high cell concentration, which would be achieved in continuous high-mass transfer bioreactors rather than in magnetically stirred bottles with a limited headspace volume. About 87% of the polymer was accumulated within the first 48h after resuspension. The 3-HV fraction in the PHA fluctuated in the range of 20 ± 1 % w w^{-1} and 27 ± 0.9 % w w^{-1} (Fig. 3c). These results were similar to those reported in the literature for the accumulation of PHBV under low valerate concentrations (100–200 mg L^{-1}). Indeed, López et al. (2018) reported that the addition of 195 mg L^{-1} of valerate during the biogas-based production of PHBV by *Methylocystis hirsuta* resulted in the inclusion of 25 % mol of 3-HV (López et al., 2018). These results were identical to those obtained by Myung et al. (2015) when supplying 100 mg/L of valerate during the conversion of methane into PHBV by *Methylocystis parvus* OBBP. In this particular study, a similar PHBV content of 73 ± 3.5 % w w^{-1} with a 3-HB:3-HV ratio of 70:30 was obtained by day 23 with the addition of valeric acid at 30% (343 mg/L) (Fig. 3e and f). The maximum PHBV productivity was 72.6 ± 2.2 g $m^{-3}d^{-1}$. Interestingly, the polymer with the highest 3-HV fraction was observed by day 13 and resulted in 53.5 ± 6.5 % w w^{-1} of PHBV with a 3-HB:3-HV ratio of 35:65 (Fig. 3f). These results suggested that the metabolic pathway was mainly directed to 3-HV accumulation during the first days after resuspension in N-free medium, and then gradually switched to the production of 3-HB. It can be concluded that the addition of valeric acid did not only favour the synthesis of 3-HV building blocks but also improved PHBV productivity. In this context, Fergala et al. (2018) found that the addition of valeric acid up to 100 mg L^{-1} increased both the co-polymer fraction and the total polymer yields, while higher valerate concentrations did not support a higher share of 3-HV and PHBV productivities dropped (Fergala et al., 2018). Several studies have reported that adding valerate above 200 mg/L reduced polymer productivity by lowering the pH to values that did not favour microbial activity (Myung et al., 2016; López et al., 2018). Interestingly, during this test series, no inhibitory effects caused by valerate were observed, likely due to the pH adjustment made prior to inoculation. Since similar results were obtained in VA-15% and VA-30% assays, the supplementation of low valerate concentrations (100–200 mg L^{-1}) was identified as the optimal scenario for triggering co-polymer accumulation. Despite the higher PHBV productivities, VA-15% tests entailed a lower consumption of the total methane and oxygen initially fed (41.7 ± 9.4 % v v^{-1} and 34.1 ± 1.6 % v v^{-1}, respectively) compared to the biotic control (Fig. 3a). Accordingly, the addition of valeric acid at 30% further reduced the methane and oxygen uptakes to 30.4 ± 5.3 % v v^{-1} and 29.2 ± 3.9 % v v^{-1} of the initial substrates present in the headspace (Fig. 3d). These slightly lower substrates consumption were likely caused by the concomitant metabolization of valeric acid.

3.3. PHBV synthesis Methylocystis hirsuta from CH_4 in a medium supplemented with M_1 synthetic VFAs mixtures

Methylocystis hirsuta was grown on methane as the main carbon and energy source and nitrate as the nitrogen source for 7 days, as previously described, up to a biomass concentration of 145 ± 7 mg TSS L^{-1} and 220 ± 29 mg TSS L^{-1} before resuspension in a nitrogen-deprived medium enriched with M_1 synthetic VFAs mixtures at 15 %C_{tot} and 30 %C_{tot}, respectively. M_1-15% and M_1-30% were composed of 66.5 and 161.5 mg L^{-1} of acetate, 57.9 and 140.5 mg L^{-1} of propionate, 39.9 and 96.9 mg L^{-1} of butyrate, 18 and 43.8 mg L^{-1} of valerate, respectively.

During the accumulation of PHBV in M_1-15% supplemented cultures, 54 ± 5 % v v^{-1} and 56 ± 4 % v v^{-1} of the total methane and oxygen initially supplied were depleted with concomitant production of 452 ± 68 g m^{-3} of CO_2 (Fig. 4a). These removal efficiencies were lower than those of *Methylocystis hirsuta* biotic controls and higher than those obtained with VA-15%. Similarly, the supplementation of *Methylocystis hirsuta* with M_1-30% under a CH_4:O_2 atmosphere resulted in lower CH_4 and O_2 consumptions of 43.4 ± 6.2 % v v^{-1} and 40.4 ± 1 % v v^{-1}, respectively (Fig. 4d). This reduction of the substrate assimilation could be associated to the simultaneous aerobic metabolization of the VFAs supplemented.

PHBV accumulation in M_1-15% supplemented cultures reached a maximum of 76 ± 1 % w w^{-1} by day 16, which was very similar to the content obtained using a valerate-supplemented medium under a CH_4:O_2 atmosphere (Fig. 4b). The maximum PHBV productivity accounted for 63.9 ± 12 g $m^{-3}d^{-1}$ and the co-polymer fraction was constant during the entire PHA accumulation period, with a 3-HB:3-HV ratio of 85:15 (Fig. 4c). Conversely, the addition of M_1 as 30% of the total carbon resulted in an inhibition of the PHBV synthesis since a maximum PHA content of 48.6 ± 2.7 % w w^{-1}, which corresponded to a maximum PHBV productivity of 103 ± 4 g $m^{-3}d^{-1}$, was obtained by day 9 with a composition of 3-HB:3-HV = 92:8 (Fig. 4e and f). The inhibitory effects observed could be due to the high concentration of organic acids in the M_1-30% mixture (VFAs tot: 443 mg L^{-1}). Indeed, the initial pH in M_1-30% supplemented cultures was lower than in the previous assays (pH = 5.6) and a large dose of NaOH was needed to adjust it to 7. Likely, adding higher amounts of NaOH could have suppressed the microbial activity. Moreover, it should be highlighted that acetic acid represented the most abundant VFAs in M_1-30% mixture, its concentration being 161 mg L^{-1}. This substrate was previously described as strongly inhibitory of methanotrophic above threshold concentrations that depended on the methanotrophic strain and culture conditions. For example, the transcription of MMO enzymes and the ability of a *M. silvestris*-spiked soil microcosms to oxidize CH_4 was repressed by the addition of about 410 mg L^{-1} of acetate, while *Methylocystis hirsuta,* was reported to tolerate acetate below ≈ 200 mg L^{-1}. These findings suggest that *Methylocystis hirsuta* was exposed to acetate concentrations in the range of inhibitory thresholds.

Overall, using M_1 synthetic mixtures triggered co-polymer accumulation, suggesting its suitability to enhance PHAs production from the anaerobic digestion of organic waste. The yields of 3-HV obtained with M_1 were lower than those obtained in the test supplemented with valeric acid VA-15% and VA-30%, likely due to the lower valerate concentrations in M_1 synthetic mixtures. In this context, valeric acid has been typically described as the most suitable additive among the various 3-HV precursors tested to synthesize PHBV as a result of its ability to activate the 3-hydroxyvaleryl-CoA (3-HVCoA) enzyme (López et al., 2018; Policastro et al., 2021).

3.4. PHBV synthesis Methylocystis hirsuta from CH_4 in a medium supplemented with M_2 synthetic VFAs mixtures

Methylocystis hirsuta was grown on methane as the main carbon and energy source and nitrate as the nitrogen source for 7 days up to a concentration of 300 ± 24 mg TSS L^{-1} and 255 ± 21 mg TSS L^{-1} before resuspension in a nitrogen-deprived medium enriched with M_2 synthetic VFAs mixtures at 15 % C_{tot} and 30 % C_{tot}, respectively. M_2-15% and M_2-30% mixtures consisted of 25.7 and 62.4 mg L^{-1} of acetate, 5.9 and 14.3 mg L^{-1} of propionate, and 67.8 and 164.8 mg L^{-1} of butyrate. No valerate was present in M_2 mixtures, which mimicked the production of VFAs from the anaerobic digestion of food waste at 55 °C.

Methane and oxygen consumptions of 41.7 ± 23 % v v^{-1} and 28.7 ± 17 % v v^{-1} were recorded in M_2-15% enriched cultures (Fig. 5a). Interestingly, the addition of M_2-30% resulted in higher CH_4 and O_2 uptakes of 55.7 ± 2.7 % v v^{-1} and 41.7 ± 3.2% v v^{-1}, contrarily to the empirical findings in section 3.3 (Fig. 5d).

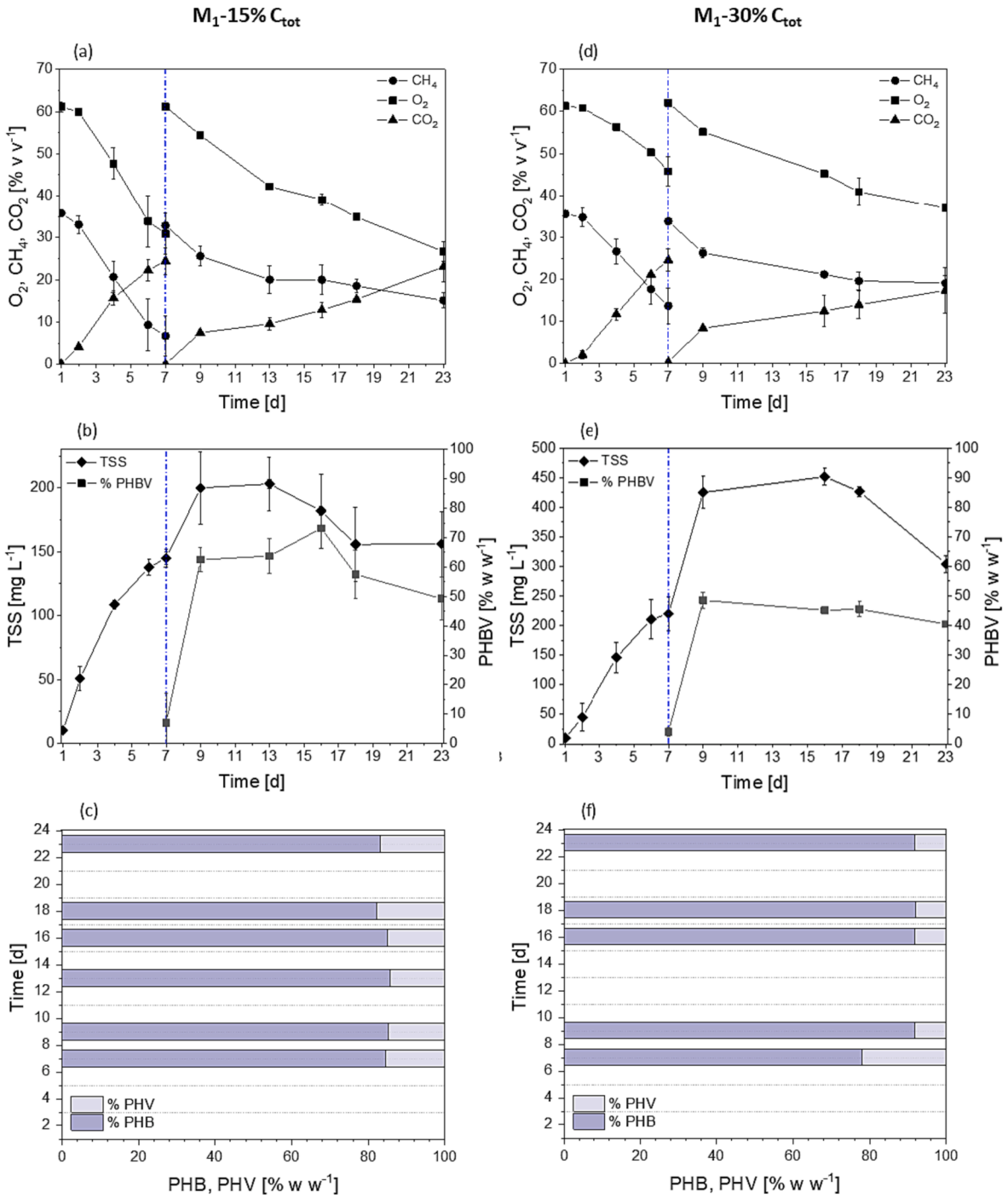

Fig. 4. Time course of methane, oxygen and carbon dioxide concentrations (a,d), and TSS and PHBV concentrations (b,e) in *M. hirsuta* grown in a medium supplemented with M_1 synthetic VFAs mixture at 15%C_{tot} (a,b,c) and 30%C_{tot} (d,e,f); PHBV=*Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)*; PHB=*Poly(3-hydroxybutyrate)*; PHV=*Poly(3-hydroxyvalerate)*.

Polymer accumulation achieved a maximum of 34.7 ± 0 % w w^{-1} by day 23 in M_2-15% supplemented cultures, with 90% of this polymer being produced during the first 48 h of nitrogen starvation (Fig. 5b). The highest PHBV productivity observed was 77.7 ± 19 g $m^{-3}d^{-1}$. Concomitantly with the higher substrate consumption compared to M_2-15% assays, higher PHBV contents and productivity of 41.8 ± 0.9 % w w^{-1} and 84 ± 14.6 g $m^{-3}d^{-1}$ were obtained during the first 2 days after resuspension with M_2-30% mixture (Fig. 5e). During this test series, a negligible amount of 3-HV was accumulated for both mixtures only during the first day of nitrogen starvation. More specifically, 1.2 and 1.56 mg L^{-1} of PHV, which accounted for 34.3 ± 13.7 % w w^{-1} and 22.9 ± 3.7 % w w^{-1} of the PHBV initial content, were obtained with M_2-15% and M_2-30% respectively. From day 8 onwards, only PHB was synthesized by *Methylocystis hirsuta* (Fig. 5f), and the composition resulted in ≈100% PHB.

Compared to the biotic controls and the other test series, the addition of M_2 mixtures reduced polymer production and did not favour the synthesis of 3-HV building blocks, thus suggesting that a not well-designed VFAs mixture can be responsible for lower process yields. More specifically, this study revealed that using mixtures deprived of valerate and with low propionate concentrations is not suitable for

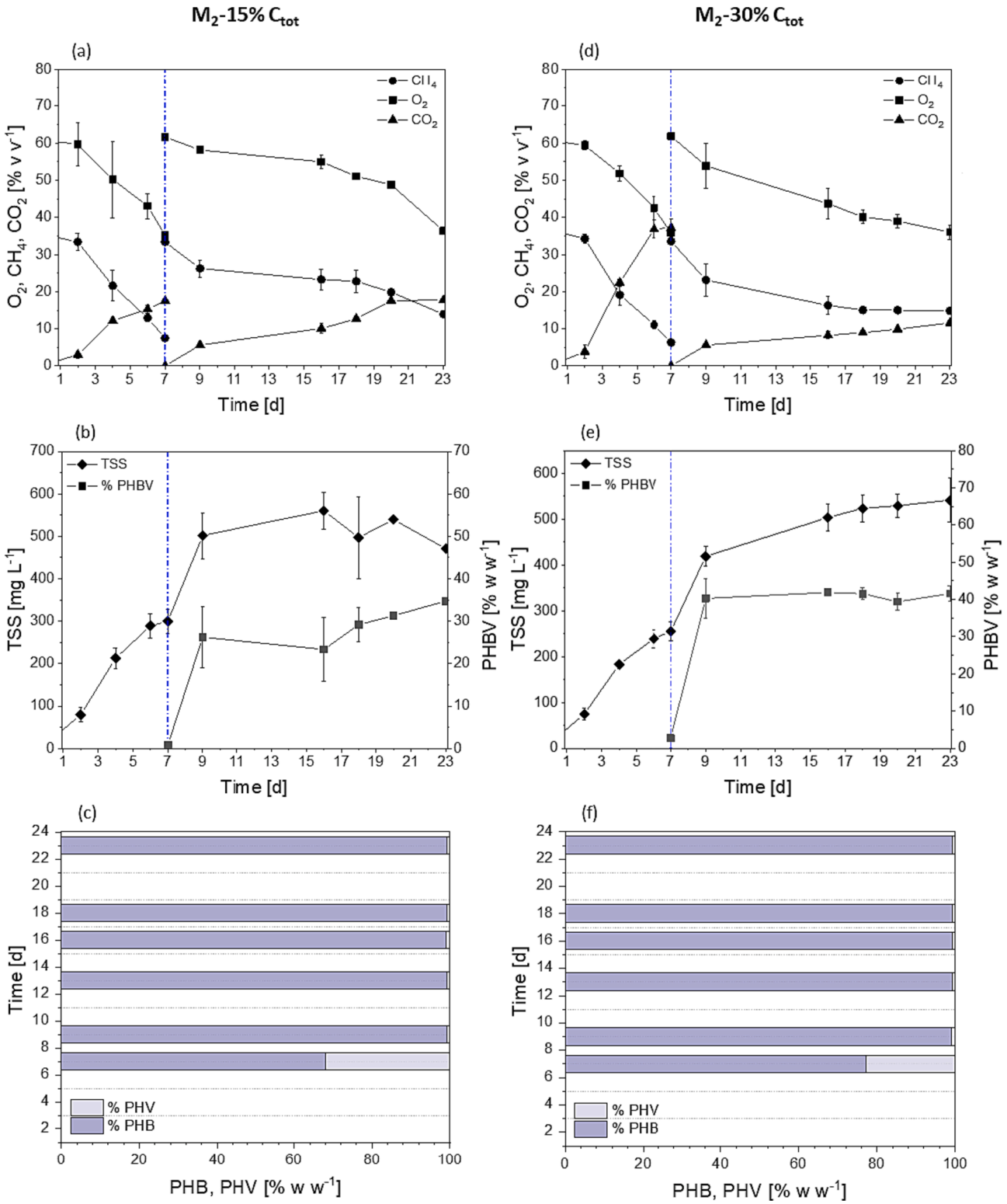

Fig. 5. Time course of methane, oxygen and carbon dioxide concentrations (a,d), TSS and PHB-co-HV concentrations (b,e) in *M. hirsuta* grown in a medium supplemented with M_2 synthetic VFAs mixture at 15%C_{tot} (a,b,c) and 30% of C_{tot} (d,e f); PHBV=*Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)*; PHB=*Poly(3-hydroxybutyrate)*; PHV=*Poly(3-hydroxyvalerate)*.

PHBV generation. In this context, the assimilation of butyric, propionic and acetic acids in methane-based cultivation requires a high energy expenditure, which hinders carbon assimilation and limits the activation of the enzymes devoted to 3-HB and 3-HV synthesis. Likely, during M_2 experiments, the 3-hydroxybutyl-CoA (3HBCoA) synthesis was favoured over the 3-hydroxyvaleryl-CoA (3HVCoA). To the best of the authors' knowledge, no similar mixtures were previously tested during the conversion of methane and VFAs into PHBV. Other experimental studies have investigated the effects of single organic acids coupled with methane bioconversion during the biosynthesis of PHAs. For instance, López et al. (2018) reported that neither acetate, butyrate, nor propionate were able to trigger co-polymer accumulation in *Methylocystis*

hirsuta cultures when fed with methane as the main carbon and energy source (López et al., 2018). Conversely, the addition of propionate in a system cultivating *Methylocystis parvus* OBBP on methane fostered the production of 8% 3-HV (Myung et al., 2016). The suitability of propionate was also reported by Luangthongkam et al. (2019), who demonstrated that 100 mg/L of propionate induced 3-HV formation in a mixed methane-utilizing culture. However, the overall PHAs productivity was very low (2.8–3.5 % w w^{-1}) (Luangthongkam et al., 2019). These findings indicated that the ability of propionate to trigger co-polymer formation depends on the strain and the concentration of propionate used. In this context, it should be highlighted that M_2 mixtures exhibited low propionate concentrations compared to M_1 mixtures, which increased 3-HV accumulation in *Methylocystis hirsuta*. It can be noted that, despite the use of biogas-derived methane has been accepted for PHAs production, the successful use of VFAs mixtures depends on their composition, and thus on the operating conditions applied during anaerobic digestion. More specifically, VFAs mixtures obtained during the thermophilic digestion of food waste, i.e. without valerate, will not be suitable to produce PHBV, while those produced at 35 °C, which are rich in valerate and contain higher concentrations of propionate, could be applied to trigger PHBV formation. In view of the above, the operating conditions during the anaerobic digestion of organic waste must support the enrichment of VFAs mixtures with high concentrations of valerate/propionate and a low share of acetate/butyrate to allow further VFAs extraction and use during the production of PHBV.

4. Conclusions

In this work, VFAs mixture and methane were used simultaneously for the first time to produce poly(3-hydroxybutyrate-co-3-hydroxyvalerate). The yields of PHBV obtained were higher than those previously reported for pure Type II methanotrophs during cultivation on methane and VFAs. Results showed that VFAs mixtures without valerate and with a low propionate did not induce 3-HV synthesis, while those containing a moderate amount of valeric acid enabled the production of PHBV. This finding suggests that further investigations should focus on generating valerate and propionate-rich streams from the anaerobic digestion of food waste to integrate biorefineries aimed at biopolymers production.

CRediT authorship contribution statement

Claudia Amabile: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Teresa Abate:** Conceptualization, Investigation. **Simeone Chianese:** Supervision, Validation, Writing – review & editing. **Dino Musmarra:** Supervision, Validation, Writing – review & editing. **Raul Muñoz:** Conceptualization, Supervision, Validation, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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