Assessing the performance of synthetic co-cultures during the conversion of methane into Poly(3-hydroxybutyrate)

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ABSTRACT

Synthetic co-cultures can enhance pollutant bioconversion performance through synergistic effects among co-existing species. In this study, the potential of *Methylocystis hirsuta* and *Methylocystis parvus* to support poly(3-hydroxybutyrate) (PHB) production in co-cultivation with *Rhodococcus opacus* and *Pseudomonas putida* under a $CH_4:O_2$ atmosphere was assessed batchwise. The metabolic activation of almost all co-cultures studied was faster than that of single strain cultures, bringing higher methane and oxygen consumption rates. Higher PHB yields were achieved when coupling *M. hirsuta* with *R. opacus* (63 % w w^{-1}) or with *R. opacus* and *P. putida* (64.4 % w w^{-1}) compared to *M. hirsuta* alone (38.5 % w w^{-1}). Interestingly, the combination of both *R. opacus* and *P. putida* with *M. parvus* reduced PHB accumulation to 42.2 % w w^{-1} compared to the content observed in *M. parvus* monocultures (62.2 % w w^{-1}) and *M parvus + R. opacus* co-cultures (66.6 % w w^{-1}).

1. Introduction

Today, methane is the second most relevant greenhouse gas (GHG) worldwide. CH_4 is more powerful than CO_2 and is responsible for approximately 0.48 W m^{-2} of direct radiative forcing [1–3]. Natural emissions, such as geological seepages, permafrost or fresh waters do not justify the current atmospheric concentration, which accounted for 1921.74 ppb at the beginning of 2023 [4]. Approximately 60 % of the CH_4 emissions are anthropogenic and originate from agriculture, oil and gas, waste and biomass burning among others [5,6]. For instance, the energy sector alone was responsible for the emission of 135 million tonnes of CH_4 worldwide in 2022 [7]. These huge amounts of methane released in the atmosphere not only affect air quality but also threaten human health and ecosystems [8]. In this context, early methane mitigation could stabilize global warming under 1.5 °C [9], highlighting the need to find aggressive abatement techniques. Several strategies to mitigate CH_4 emissions have been explored in the last decades, with CH_4 conversion into value-added products being identified as the most promising approach [10,11]. In fact, methane could represent an effective alternative feedstock for biological processes currently using expensive carbon sources [12–14].

The microorganisms responsible for the conversion of methane, known as methanotrophs, were observed for the first time by Sönghen in

1906 and are nowadays divided into two assemblages depending on their metabolic pathway: Type I (which belong to γ-Proteobacteria class) and Type II (α-Proteobacteria class), which use the ribulose monophosphate and the serine pathways, respectively [14,15]. The main feature of methanotrophic bacteria is the use of methane monooxygenase enzymes (MMOs), which catalyse the oxidation of methane to methanol [16]. These enzymes could be either particulate (pMMO) or soluble (sMMO) [15]. The assimilation of CH_4 occurs through its oxidation by MMOs to methanol, the subsequent conversion of methanol to formaldehyde and the oxidation of formaldehyde to formate or its conversion into CO_2 [6].

Type II methanotrophs of the genera *Methylocystis* and *Methylosinus* are regarded as a promising methane bioconversion platform due to their ability to accumulate significant amounts of Poly-hydroxyalkanoates (PHAs) using methane as the sole carbon and energy source under nutrient-limited conditions [17,18]. PHAs are biobased polyesters that can be produced biologically by many microorganisms under metabolic stress conditions and are accumulated as intracellular granules that serve as carbon and energy reserve [19]. Overall, PHAs are insoluble in water but soluble in several solvents, exhibit good resistance to hydrolytic attack, are resistant to UV, biocompatible, biodegradable and behave as piezoelectric materials [20]. These properties make PHAs a valid alternative to fossil-based plastics. *Methylocystis parvus* and

Methylocystis hirsuta have been typically reported to accumulate up to 50 % w w^{-1} of Poly(3-hydroxybutyrate) (PHB) and Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB-co-HV) when using methane as the sole carbon and energy source [21–24].

However, despite the successful production of PHAs at a laboratory scale, the transition to pilot or industrial scale still faces several challenges [25]. These limitations include, for instance, the difficulty in establishing the optimal operating conditions and enhancing the methane mass transfer capacities. Previous research has been focused on analysing key factors such as temperature, $CH_4:O_2$ ratio, and nitrogen source to optimize the growth of *Methylocystis hirsuta* and the subsequent PHAs accumulation under nutrient limitation [26]. Many efforts have also been carried out to enhance mass transfer by developing innovative bioreactors. Bubble column reactors and Taylor flow reactors equipped with internal gas recycling were used for producing PHAs from *Methylocystis hirsuta* and mixed consortia, achieving high methane utilisation efficiencies up to ≈73 % and ≈60 %, respectively [27,28]. Nevertheless, in some cases, the release of undesired metabolites during methane biodegradation, which inhibited the metabolism of *Methylocystis hirsuta* and undermined its microbial stability, was observed [21].

In this context, an alternative to pure methanotrophic cultures for the efficient conversion of methane into PHAs is the use of well-designed synthetic co-cultures [29]. In fact, the *ad-hoc* use of a microbial partner could induce a synergistic effect among different strains by reducing metabolic burdens and creating an optimal environment for the biosynthesis of bioproducts [29,30]. Indeed, a reduction of the inhibitory effects caused by the release of undesired byproducts was observed when using well-designed co-cultures during the production of *cis,cis*-muconic acid and 4-hydroxybenzoic acid from a mixture of glucose/xylose [30]. In this context, the design of synthetic co-cultures should be rational and grounded on the evaluation of the extracellular metabolites of the species considered. In this particular case, Type II methanotrophs, for instance, produce formaldehyde and formate in the metabolic pathway supporting PHAs accumulation [31]. The cytotoxic effects of these inhibitory metabolites limit the effective design of a co-culture to a few strains. *Pseudomonas* and *Rhodococcus* species, which are also PHA producing microorganisms, are potential candidates for methanotrophs-based co-cultures since their formaldehyde and formate dehydrogenases make them resistant to low-medium concentrations of these compounds [32,33]. For instance, *Pseudomonas putida* was reported to tolerate up to 1.5 mM of formaldehyde, while *Rhodococcus* species were able to completely remove consecutive doses formaldehyde from both synthetic and industrial wastewaters at a concentration of 20 mg L^{-1} [32,33]. Unfortunately, although the use of synthetic co-cultures has been explored in industrial applications such as carbon monoxide bioconversion or for the bioconversion of methane into mevalonate, the use of methanotrophic co-cultures to boost PHAs accumulation under high methane loads has not been explored to date [29].

This work was designed to serve as a pioneering study for assessing the ability of synthetic co-cultures to support a synergistic effect during the accumulation of PHB when using methane as the sole carbon and energy source. *Methylocystis parvus* and *Methylocystis hirsuta* were used as the main Poly(3-hydroxybutyrate) producers, while *Pseudomonas putida* and *Rhodococcus opacus* were chosen as partner microorganisms. The PHB production yields of the pure strains and the rationally designed co-cultures were compared to the performance of a mixed methanotrophic consortium (MMC).

2. Materials and methods

2.1. Chemicals

Chemicals for culture medium preparation were purchased from PANREAC AppliChem (Barcelona, Spain) except for $CoCl_2$, FeEDTA, Cl_2Ni $6H_2O$, $ZnSO_4$ $7H_2O$, $FeSO_4$ $7H_2O$, H_3BO_3, $NiCl_2$ $6H_2O$, Na_2HPO_4 12 H_2O, Na_2MoO_4 $2H_2O$, which were purchased by Sigma Aldrich and

KNO_3 by Labkem (Barcelona. Spain). Chloroform (≥99.8 %), 1-propanol (99.7 %), benzoic acid (99.5 %), hydrochloric acid (37 % w v^{-1}) and Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB-co-HV) with 12 % mol 3-HV (99.99 %) were used for PHB extraction/measurements and purchased from Sigma Aldrich. O_2 (99.5 %) and CH_4 (99.5 %) were acquired from Abelló Linde S.A. (Spain) and Carburos Metalicos (Spain), respectively.

2.2. Strains and culture medium

Methylocystis parvus OBBP (Biopolis S.L., Valencia, Spain), *Methylocystis hirsuta* CSC1 (Leibniz-Institut DSMZ, Germany) and a *Methylocystis*-enriched mixed consortium [27] were grown in a mineral salt medium (NMS) containing 0.2 g L^{-1} $CaCl_2$ $2H_2O$, 1.0 g L^{-1} KNO_3 and 1.1 g L^{-1} $MgSO_4$ $7H_2O$, 1 mL L^{-1} of a trace element solution as previously described by Rodriguez et al. (2020) and 10 mL L^{-1} of a buffer solution containing 72 g L^{-1} Na_2HPO_4 $12H_2O$ and 26 g L^{-1} KH_2PO_4 to adjust the pH to 6.8 [21].

Pseudomonas putida KT2440 and *Rhodococcus opacus* DSM 43205 were purchased from the Leibniz Institute (Germany) and grown in M9 mineral salt medium consisting of (g L^{-1}): 7.52 Na_2HPO_4 $2H_2O$, 3 KH_2PO_4, 0.5 NaCl, 0.5 NH_4Cl, 4 $C_6H_{12}O_6$, 0.246 $MgSO_4$ $7H_2O$, 0.044 $CaCl_2$ $2H_2O$ and vitamins (1 mL biotin and 1 mL thiamine solutions). 10 mL L^{-1} of a trace element solution with the following composition (g L^{-1}) were also added: 5 EDTA, 0.83 $FeCl_3$ $6H_2O$, 0.084 $ZnCl_2$, 0.013 $CuCl_2$ $2H_2O$, 0.01 $CoCl_2$ $2H_2O$, 0.01 H_3BO_3, 0.0016 $MnCl_2$ $4H_2O$.

2.3. Experimental procedures

2.3.1. Inocula preparation

Stock cultures of *Methylocystis hirsuta* CSC1, *Methylocystis parvus* and the mixed methanotrophic consortium were activated according to the procedure described by Rodriguez et al. (2020) [21]. Sterile 125 mL serum bottles containing 50 mL of NMS medium were inoculated at 10 % (v v^{-1}), capped with butyl-rubber stoppers and crimp-sealed before being incubated in an orbital shaker at 200 rpm and 25 °C for 6 days. The headspace was filled with a mixture of $O_2:CH_4$ (2:1 v v^{-1}), which was replaced every 48 h twice. In order to work under sterile conditions during headspace replacement, oxygen was filtered (0.2 μm) while flushing for 5 min, and the oxygen atmosphere (25 mL) was replaced with methane using a 50 mL gastight syringe (Hamilton 1050 TLL, USA).

Pseudomonas putida and *Rhodococcus opacus* were grown in 125 mL serum bottles containing 20 mL of M9 mineral salt medium inoculated at 10 % (v v^{-1}) under strictly sterile conditions, capped with butyl-rubber stoppers and agitated in a multipoint stirrer (Thermo Scientific™) at 250 rpm and 25 °C for 2 days.

2.3.2. Design, growth and PHB accumulation in methanotrophs-based co-cultures

Sterile 2.15 L serum bottles containing 0.5 L of NMS were inoculated with 10 mL of fresh pure Type II methanotrophs or the mixed methanotrophic consortium under a $CH_4:O_2$ atmosphere (33.3:66.6 v v^{-1}). *M. hirsuta* (initial OD_{600} of 0.16) and *M. parvus* (initial OD_{600} of 0.06) were used as biotic control tests, while a mixed methanotrophic consortium (MMC) (2 % v v^{-1}, initial OD of 0.04) was used to compare the PHB yields to those of synthetic co-cultures. When methane concentration became negligible and substrate assimilation was no longer observed, the biomass was harvested by centrifugation (4,200 rpm, 10 min) using a Sorvall X PRO series Centrifuge (Thermo Scientific ™) and resuspended in 2.15 L serum bottles containing 0.5 L of nitrogen-free salt medium (NFSM) to assess the ability of the cultures to accumulate PHB. The bottles were crimp-sealed, filled with a methane/oxygen mixture as described above and incubated at 350 rpm and 25 °C using multipoint stirrers (Variomag, Thermo Fisher Scientific, USA). It should be noted that, during this phase of the experiment, a higher RPM compared to the step of inocula preparation was selected in order to achieve a higher
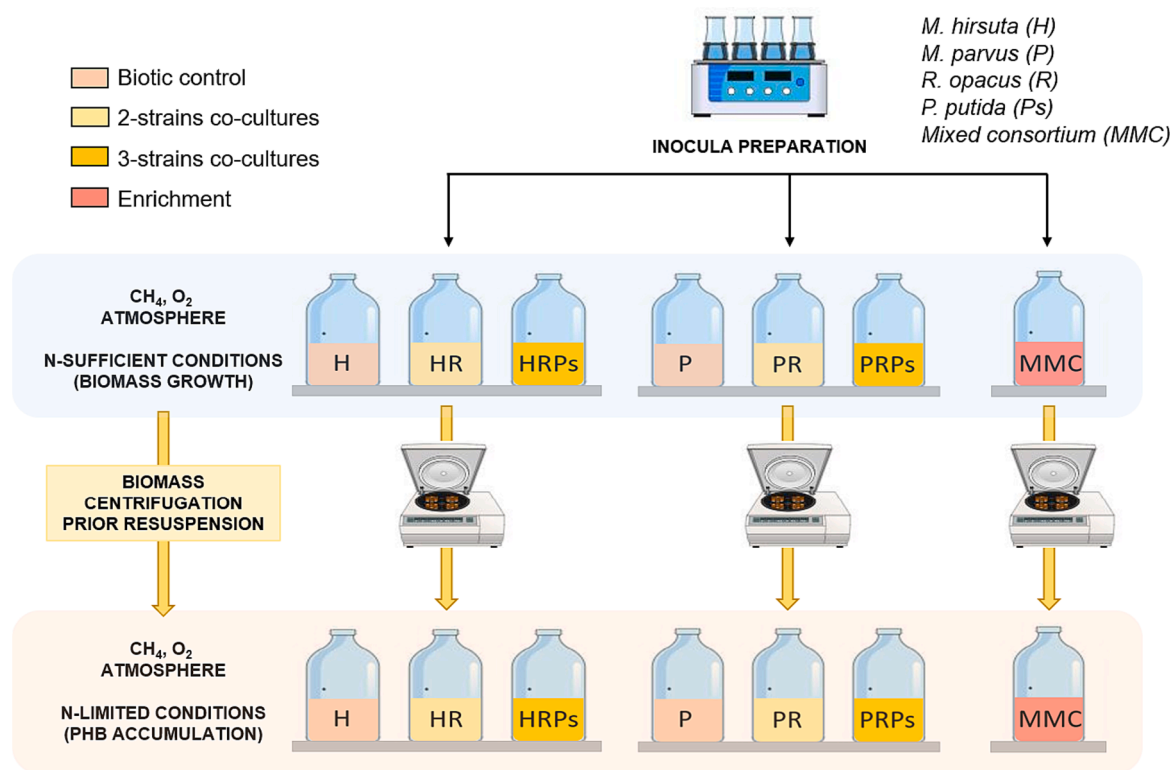
Fig. 1. Experiments conducted to assess the influence of co-culturing on methane biodegradation and PHAs accumulation.

mass transfer of methane and oxygen to the liquid phase. The headspace composition, the OD_{600}, Total Suspended Solids (TSS) and PHB content were periodically monitored. All experiments were conducted in duplicate.

Synthetic co-cultures tests were also performed in duplicate to assess the ability of pure Type II methanotrophs to grow and accumulate PHB when combined with one or two heterotrophs. The following combinations were investigated: *M. hirsuta + R. opacus* (HR), *M. hirsuta + R. opacus + P. putida* (HRPs), *M. parvus + R. opacus* (PR), *M. parvus + R. opacus + P. putida* (PRPs). To promote culture growth, 2.15 L bottles containing 0.5 L of mineral salt medium were inoculated with 10 mL of each strain, closed with chlorobutyl-rubber stoppers and sealed with aluminium caps. The inoculum culture broth of *R. opacus* and *P. putida* was centrifuged twice at 10,000 rpm for 10 min prior inoculation: the first centrifugation was aimed at removing most of the glucose and ammonia residual from the first state of cultivation and harvesting the cells from the culture broth; the second centrifugation was used to remove the trace levels of glucose and ammonia. The initial OD_{600} of HR, HRPs, PR and PRPs accounted for 0.196, 0.21, 0.1 and 0.115, respectively. The headspace of the bottles was filled with a methane-oxygen atmosphere (33.6:66.7 v v^{-1}) prior incubation under continuous stirring at 350 rpm and 25 °C in a multipoint stirrer (Variomag, Thermo Fisher Scientific, USA). The biomass concentration (determined from OD_{600} measurements) and headspace concentrations were periodically monitored.

At the end of the growth phase, i.e. when methane and oxygen consumption became negligible, the co-culture broths were centrifuged at 4,200 rpm for 10 min to harvest and resuspend the pellet in 2.15 L bottles containing 0.5 L of sterile NFSM. The atmosphere was filled again with a mixture of oxygen and methane (2:1 v v^{-1}), and the cultures were incubated under continuous stirring at 350 rpm and 25 °C until methane removal was negligible. The biomass concentration (determined from OD_{600} measurements), PHB content and headspace concentrations were periodically monitored. An overview of the experimental test series is shown in Fig. 1.

2.4. Analytical methods

CH_4, CO_2 and O_2 concentrations in the headspace of the bottles were monitored every 48 h using a Bruker 430 GC-TCD (Bruker Corporation, Palo alto, USA) equipped with a CP Molsieve 5A and a CP-PoraBOND Q columns. The oven, the injector and the detector were kept at 45, 150 and 200 °C, respectively. Culture absorbance at 600 nm was measured every 48 h using a UV-2550 spectrophotometer (Shimadzu, Japan). TSS were measured on a dry basis according to the 2540 standard method [34]. PHAs were extracted and quantified through 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. Samples for PHAs measurements were prepared in triplicate: 1.5 mL of culture broth was centrifuged at 10,000 rpm for 10 min (Spectrafuge^{TM} 24D, Labnet) and stored at −20 °C until use. For PHAs extraction, 1 mL of 1-propanol-HCl solution (80:20 % v v^{-1}) and 2 mL of chloroform were added to the collected samples prior digestion for 4 h at 100 °C. Benzoic acid and PHB-co-HV (12 %mol 3-HV) were used as internal and external standards, respectively. The procedure for polymer quantification and identification was used as described elsewhere [22]. Note that the GC–MS analysis also allowed the characterization of the polymer though the identification of the monomers forming the PHAs produced.

3. Results and discussion

3.1. Microbial growth and PHB accumulation in M. hirsuta-based co-cultures

The time required for the metabolic activation of *M. hirsuta*, which was intended as the time needed to start observing substrate consumption, accounted to 15 days. The strain grew up to 540 ± 57 mg TSS L^{-1} (OD_{600} = 2.3) by day 22 at an average growth rate of 45 ± 28 mg TSS L^{-1} d^{-1} (Fig. 2b). Note that the growth rate of the cultures was strongly influenced by the poor gas–liquid mass transfer in the magnetically
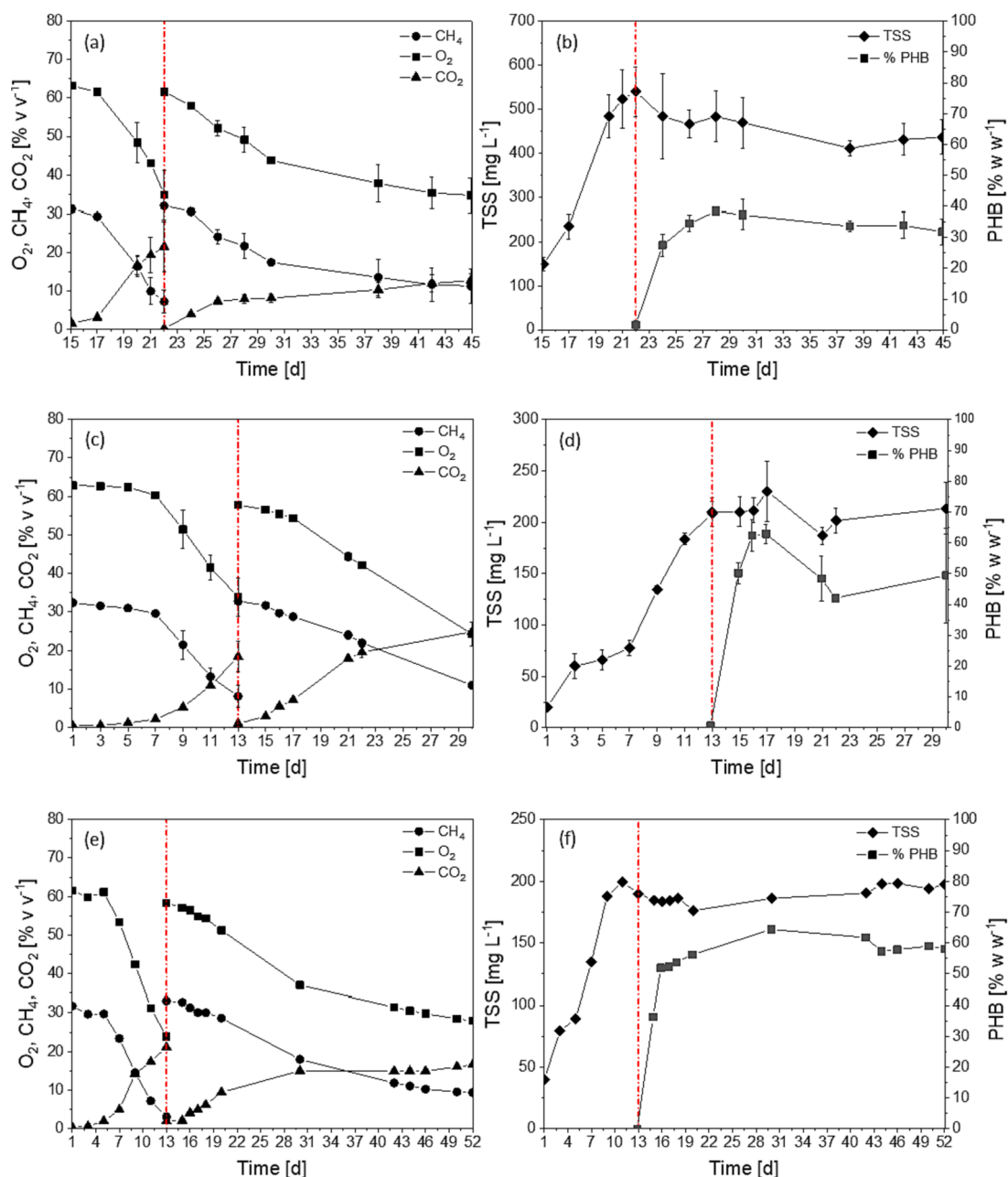
Fig. 2. Time course of the concentration of methane, oxygen, carbon dioxide, TSS and PHB in cultures of *M. hirsuta* (a-b), *M. hirsuta* + *R. opacus* (c-d) *and M. hirsuta* + *R. opacus* + *P. putida* (e-f).

stirred bottles, while at industrial scale the higher volumetric gas–liquid coefficient would prompt higher biomass growth rates [35]. During the growth phase, 77 ± 9 % v v^{-1} and 44 ± 10 % v v^{-1} of the total methane and oxygen initially supplied were consumed, respectively (Fig. 2a). The medium oxygen to methane consumption ratio calculated for a 24 h period of cultivation was 1.43. Despite this ratio was slightly lower than those reported in the literature, since 1.5–2 mol of O_2 per mole of CH_4 were commonly consumed during the cultivation of *M. hirsuta* [26], the growth performance was not compromised. At the end of the growth phase, *M. hirsuta* cultures, which contained 1.53 % w w^{-1} of PHB, were resuspended in a nitrate free medium to promote PHB accumulation. The highest rate of biopolymer production was recorded within the first two days of incubation (61.1 mg PHB L^{-1} d^{-1}), resulting in a PHB content of 27.5 ± 4 % w w^{-1}. Approx. 1.5 mol of O_2 were used per mole of methane oxidized under N limiting conditions. From day 25 onwards, the polymer production rate decreased to 13.5 ± 1 mg PHB L^{-1} d^{-1} and

the maximum PHB content (38.5 ± 1 % w w^{-1}) was obtained by day 28. Six days after pellet resuspension, the fraction of PHB stored remained constant, and methane consumption became negligible. This behaviour was previously reported during the cultivation of *M. hirsuta* on methane: about 80 % of the total PHB produced was accumulated within the first 48/72 h and remained constant at 45 % w w^{-1} from day 5 onward [22].

Co-cultures containing *R. opacus* (HR) and *R. opacus* + *P. putida* (HRPs) as partner microbes showed a significant reduction in the activation time (CO_2 production, methane and oxygen consumption started by day 7 and 5, respectively) (Fig. 2c and 2e). Conversely, the average growth rate decreased to 21.8 ± 8 mg TSS L^{-1} d^{-1} and 15.9 mg TSS L^{-1} d^{-1} for HR and HRPs cultures, respectively (Fig. 2d and 2f). After 13 days of continuous growth, the optical density of the cultures containing both *M. hirsuta* and *R. opacus* was 1.7 ± 0, which corresponded to 208 ± 12 mg TSS L^{-1}, while co-cultures also containing *P. putida* reached a maximum concentration of 190 mg TSS L^{-1} (OD_{600}:1.6). It is worth
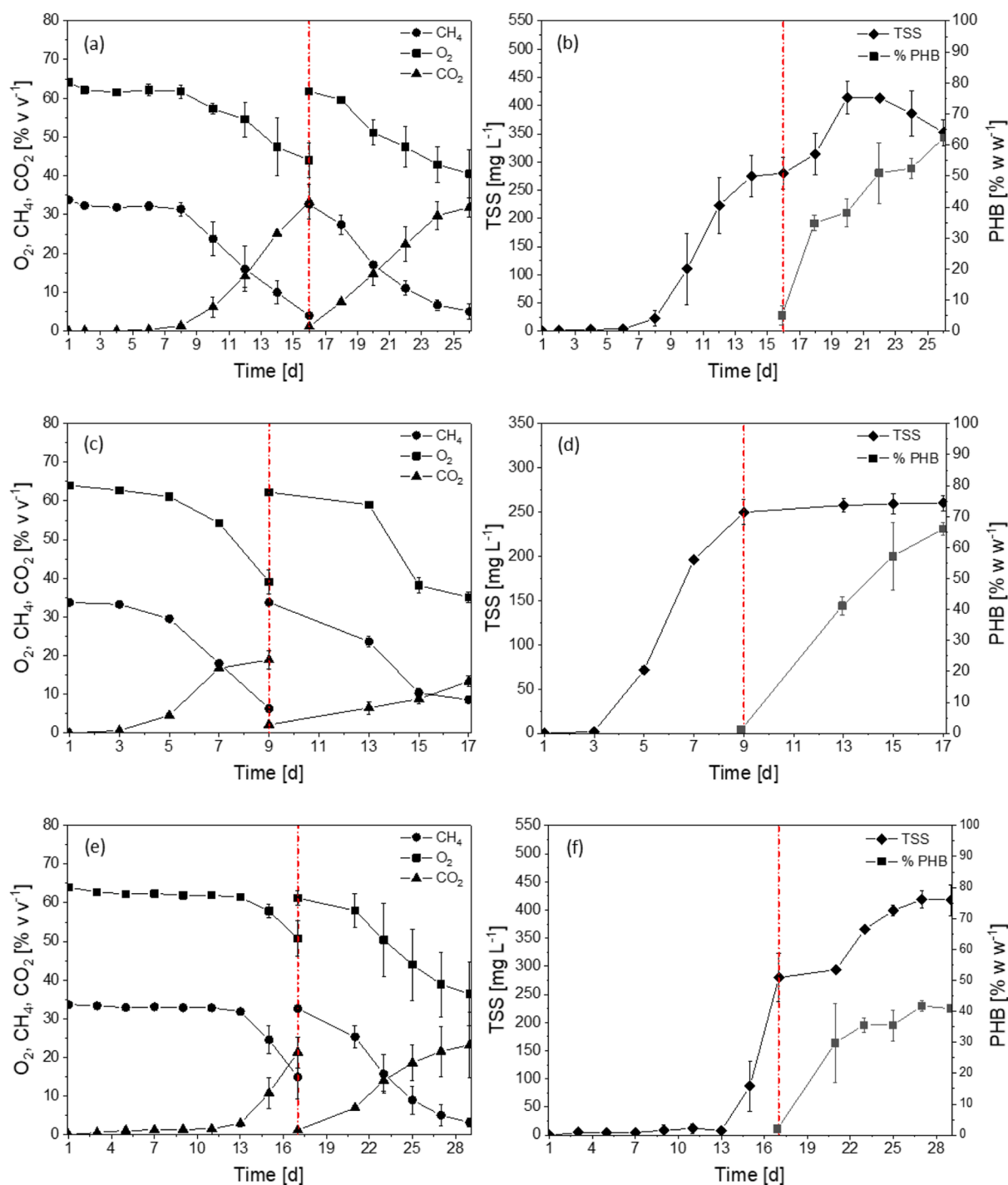
Fig. 3. Time course of the concentration of methane, oxygen, carbon dioxide, TSS and PHB in cultures of *M. parvus* (a-b), *M. parvus + R. opacus* (c-d) *and M. parvus + R. opacus + P. putida* (e-f).

noting that, during this phase, the non-methanotrophic strain survived feeding on the organic metabolites secreted by *Methylocystis* species, and the addition of glucose was not needed. Interestingly, both the growth rate and biomass productivity decreased in HR and HRPs systems: it is likely that the strains involved competed for oxygen during cultivation under normal metabolic conditions. This behaviour was more evident as the number of strains involved increased, where an environment with more strain-to-strain interactions was generated. Methane and oxygen consumptions in HR-cultures were very similar to those observed in *M. hirsuta* control (75 ± 9 % v v^{-1} and 46 ± 8 % v v^{-1}, respectively), while slightly higher depletions of 90 % v v^{-1} CH$_4$ and 61 % v v^{-1} O$_2$

(CH$_4$:O$_2$ molar ratio 1:1.4) were obtained with HRPs-cultures during the growth phase. At the end of the growth phase, HR and HRPs co-cultures contained 1.54 % w w^{-1} and 0 % w w^{-1} of PHB, respectively. On day 13, both synthetic co-cultures were resuspended in a nitrate-free medium to promote PHB synthesis. Maximum accumulation rates of 52 mg PHB L^{-1} d^{-1} and 33.4 mg PHB L^{-1} d^{-1} were observed during the first two days in HR and HRPs, respectively. In this context, note that despite *P. putida* is known to be a mcl-PHAs producer and its co-culturing with Type II methanotrophs could have led to blend PHAs, only PHB was detected during GC–MS analysis. Approximately 1.3 and 1.4 mol of oxygen were used to oxidize 1 mol of methane during PHB production in HR and

HRPs cultures, respectively. The results obtained in terms of substrate consumption in *M. hirsuta* monoculture and HR co-cultures show very similar methane and oxygen removals, while the addition of *P. putida* led to higher substrates depletion. It is also worth noting that lower $O_2:CH_4$ molar consumption ratios were observed during co-cultivation, which could highlight the synergism among the species involved. This finding agrees with the literature, where co-cultures of methanotrophs and heterotrophs showed a higher methane removal efficiency compared to single strain cultures, likely due to the enhanced expression of pMMO genes. At this point, it is worth noting that, although methanotrophs have been reported to have metabolic interactions with several heterotrophs, these kinds of mutual effects are strongly strain dependent. In this study, the addition of *R. opacus* alone or coupled with *P. putida* in *M. hirsuta*-based co-cultures led to a similar PHB accumulation, thus suggesting the possibility of cost-effectively using both strains for improving the performance of methanotrophic monocultures. Indeed, the highest PHB content in *M. hirsuta* + *R. opacus* cultures was obtained by day 17 (63 ± 3 % w w^{-1}) at a production rate of 14.1 mg PHB L^{-1} d^{-1}. A similar behaviour was observed when *P. putida* was added to the co-culture: the PHB accumulation rates decreased from day 15 onwards with a maximum PHB content of 64.4 % w w^{-1} reached by day 31 (Fig. 2e and 2f). Overall, the PHB accumulation capacity of the proposed co-cultures, with respect to the total suspended solids, is 2-fold higher than that of *M. hirsuta* alone. It is likely that, under metabolic stress conditions, the co-cultivation with heterotrophs prompted a very specific activity by enhancing the PHB production pathway. Indeed, an effective transfer of metabolites, organic compounds or macromolecules, such as proteins and RNA, may occur in co-cultures causing the strains to potentially influence each other's metabolism directly. Moreover, the direct physical interaction between different species may prompt a participant to carry out a particular activity: the higher PHB production and substrate consumptions observed during co-cultivation, for example, could be related to the quick activation of the pMMO genes, which are responsible for the high cell-specific activity of the methanotrophic population.

Although examples of synergistic co-cultures have already been reported in the literature, no methanotrophic-heterotrophic co-cultures aimed at fostering methane conversion to PHB have been investigated to date. Thus, *Synechoccus elongatus* was reported to fix CO_2 carbon in the form of sucrose, which was used by sucrose-metabolizing heterotrophs to produce PHB in co-cultivation systems [36]. Similarly, an increased PHB production was observed when growing co-cultures of *Azotobacter chroococcum* and *Bacillus megaterium* or *Ralstonia eutropha* and *Lactobacillus delbrueckii* on glucose and fructose. Indeed, the co-cultivation of *L. delbrueckii* increased PHB yields by 19 % compared to the stand-alone cultivation of *R. eutropha* [37]. Finally, the benefits of bacterial co-culturing were also reported in other fields, such as biogas conversion or the production of enzymes, antimicrobial substances and food additives [38,39].

Interestingly, using co-cultures led to lower yields in terms of biomass production, but the pellet obtained at the end of the accumulation phase was 2-fold richer in PHB than in the case of pure *M. hirsuta* cultures. This scenario could be highly relevant during the implementation of some PHB extraction process techniques, where the recovery and purity yields are dependent on the initial polymer content [40,41]. In this context, the abundance of non-PHAs materials in the cells at similar PHB concentrations could reduce the purity of the polymer recovered. Indeed, Yang and co-workers (2011) reported that *R. eutropha* and *E. coli* cells with high PHA content (82 % of the cell dry weight) supported a higher purity extraction regardless of the method applied compared to cells with lower PHA contents of 45 % and 33 %, respectively [40].

3.2. Microbial growth and PHB accumulation in M. parvus-based co-cultures

The metabolic activation of *M. parvus*-based biotic controls (i.e. substrates consumption, CO_2 generation) occurred by day 8. Biomass concentration reached 280 ± 28 mg TSS L^{-1} by day 16, which corresponded to an OD_{600} of 1.9, at an average rate of 42 ± 16 mg TSS $L^{-1}d^{-1}$ (Fig. 3b). Methane and oxygen consumption accounted for 88 ± 3 % v v^{-1} and 31 ± 7 % v v^{-1}, respectively (Fig. 3a), while the PHB content was 7.35 % w w^{-1} by the end of the growth phase. Cultures were resuspended in a nitrate-free mineral salt medium by day 16, and a maximum PHB content of 62 ± 0.9 % w w^{-1} was observed by day 26. The highest polymer accumulation rate occurred during the first two days after pellet resuspension (40.4 mg PHB $L^{-1}d^{-1}$) and then decreased to 25 ± 1.8 mg PHB $L^{-1}d^{-1}$ between days 18–22 (Fig. 3b) [22]. Similarly, *M. parvus* was reported to accumulate 50 % w w^{-1} of PHB within the first 48 h of cultivation when methane was used as the sole carbon source [23].

Cultures containing both *M. parvus* and *R. opacus* were metabolically active after only 2 days of cultivation and reached a concentration of 250 ± 14 mg TSS L^{-1} by day 9 ($OD_{600} = 1.7$) at an average rate of 41 ± 19 mg TSS L^{-1} d^{-1} (Fig. 3d). Approx. 81 ± 3 % v v^{-1} and 39 ± 5 % v v^{-1} of the methane and oxygen initially supplied were consumed by the end of the growth period (Fig. 3c). PHB content was 0.96 % w w^{-1} by the end of the growth period. On day 9, cultures were resuspended in nitrogen-deprived medium to promote PHB accumulation. The maximum PHB content was obtained by day 17 and accounted for 66.6 % w w^{-1} of the TSS. Similarly to the monoculture assay, the PHB production rate was the highest during the first four days after resuspension (51.62 mg PHB $L^{-1}d^{-1}$) and then decreased progressively to 21.3 mg PHB L^{-1} d^{-1} and 11.4 mg PHB L^{-1} d^{-1} by day 15 and 17 respectively (Fig. 3d). During the PHB accumulation phase, 1.9 mol of oxygen were used to oxidize 1 mol of methane. Interestingly, despite the rapid activation of HR-cultures, the use of *R. opacus* as a single partner microbe resulted in a very similar TSS concentration and PHB content compared to the monocultures of *M. parvus*, thus suggesting that the direct physical interaction boosted methane-oxidation activity by promoting the expression of pMMO genes.

Unlike the assays conducted with HRPs, cultures of *M. parvus* containing both *R. opacus* and *P. putida* were likely inhibited by the presence of *P. putida*. Indeed, the time for activation of PRPs increased to 13 days, and the consumption of the substrate decreased to 56 ± 17 % v v^{-1} CH_4 and 21 ± 7 % v v^{-1} O_2 when compared to the biotic control and PR-cultures (Fig. 3e). Interestingly, the addition of the third microbial partner induced an antagonistic effect in HRPs-cultures, leading to an energy-intensive process in which 2.7 mol of oxygen were needed for the oxidation of 1 mol of methane. No difference compared to the *M. parvus* controls and PR-cultures was observed in terms of total suspended solids by the end of the growth phase, where 280 ± 42 mg TSS L^{-1} containing 2.8 % w w^{-1} of PHB (OD_{600}:1.45) (Fig. 3f) were recorded by day 17. These results suggest that the interactions between *P. putida* and *M. parvus* could delay the activation mechanisms and reduce substrate assimilation during culture growth but do not inhibit cell reproduction. Conversely, significant inhibition was observed during the accumulation of PHB since the highest polymer content (42 ± 2 % w w^{-1}) was ≈ 20 % lower than in the cultures of *M. parvus* and *M. parvus* + *R. opacus*, and the maximum PHB production rate (25 ± 1.6 mg PHB L^{-1} d^{-1}) was 2 times lower than the rates achieved during PHB accumulation in *M. parvus* controls and PR-cultures (Fig. 3f). It can be hypothesized that *P. putida* competed for oxygen, thus limiting methane oxidation by the methanotrophs. In this regard, a study assessing Poly(3-hydroxybutyrate) production during the cultivation of *Methylosinun thricosporium OB3b* coupled with a phototrophs community, including *Pseudomonas* species, demonstrated the crucial role of O_2 [42]. The relative abundance of *P. putida* in the 3-strains culture could therefore justify the observed inhibitory effect [43]. This was also previously shown during the
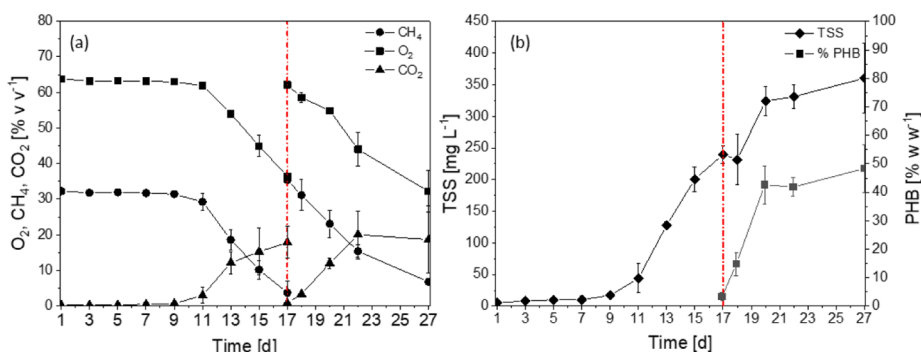
Fig. 4. Time course of the concentration of methane, oxygen, carbon dioxide (a), TSS and PHB (b) during the cultivation of a mixed methanotrophic consortium.

conversion of methane into mevalonate when co-cultivating Type I methanotrophs with *Escherichia coli* [29]. In this study, the authors demonstrated that the variation of the communities ratio strongly affected microbial growth since ratios of 5:1 and 10:1 allowed the growth of both strains, while a ratio of 1:1 did not supported methanotrophic growth [29]. Therefore, it was impossible to confirm whether *P. putida* was unsuitable for enhancing PHB production when coupled with *M. parvus* and *R. opacus* or if the microbial ratio used in this work mediated an unfavourable growth environment by suppressing methanotrophic activity.

3.3. Microbial growth and PHB accumulation in MMC

Methane assimilation started after 11 days of cultivation, and the cultures grew up to 240 ± 14 mg TSS L^{-1} (OD$_{600}$:1.56) with an initial PHB content of 4.4 % w w^{-1} at an average rate of 33 ± 12 mg TSS L^{-1} d^{-1} (Fig. 4a). From day 11 to 17, approx. 88 ± 10 % v v^{-1} and 43 ± 2 % v v^{-1} of the methane and oxygen initially supplied were consumed. PHB accumulation started on day 17 and resulted in a maximum polymer content of 48 ± 8 % w w^{-1} by day 27 (Fig. 4b). The highest productivity of PHB was observed within the first 48/72 h and accounted for 51.9 mg PHB L^{-1} d^{-1}. In this period, ~ 1.6 mol of oxygen were used for methane oxidation during PHB accumulation.

Myung et al. (2015) reported that a *Methylocystis*-dominated consortium accumulated up to ≈ 43 % w w^{-1} of PHAs under nutrient-limited conditions using methane as the sole carbon and energy source [24]. Similarly, PHAs contents of 59.4 % and 54.3 % were obtained using a methane-oxidizing consortium enriched through media-based selection [44]. A maximum PHAs content of 38 % w w^{-1} was recorded in a Taylor flow reactor operated with the same consortium used in this study [27]. The higher PHAs yields reported in this work could be related to the relative abundance of Type II methanotrophs, the higher CH$_4$ concentrations and the batch nature of the cultivation. Indeed, mixed methanotrophic cultures are dynamic, and the population structure varies with several factors, such as temperature, pH, substrate concentration, dilution rate, etc. In this context, Cattaneo et al. [27] reported that the Type II fraction of methanotrophs decreased from 51 % to 38 % by the end of the operation in the reactor. Thus, despite the advantages of mixed methanotrophic consortia, the lack of microbial stability of MMC might imply a high variability of the PHAs production and quality, thus hampering their applicability at a large scale. In this context, applying co-cultures would entail an easy understanding of the interaction mechanisms and allow a more robust control of the species involved in the methane bioconversion process. Indeed, it should be noted that the occurrence of antagonistic/synergistic effects in MMC or co-cultures is a function of the number of microorganisms [45]. Moreover, MMC or co-cultures with more than 2 strains experience more complex microbial interactions, also influenced by external environmental factors. These mutual effects are difficult to control, which might explain why only 2-strains co-cultures have been studied to date [45]. In our study, except

for the case of PRPs, the use of synthetic co-cultures induced higher PHA yields compared to the MMC. This phenomenon was likely due to the high accumulation capacity of the pure strains used and the favourable cultivation conditions. A similar finding was previously reported in methanotrophic co-cultures devoted to the production of methanol, where yields of 57.5 % were observed in the co-culture compared to 45 % in an open mixed consortium [46].

4. Conclusions

In this work, the production of Poly(3-hydroxybutyrate) from methane via the co-cultivation of methanotrophs and heterotrophs was assessed for the first time, on the basis of authors' knowledge. Among all the combinations studied, only co-cultures of *R. opacus*, *P. putida* and *M. parvus* resulted in lower PHB yields, likely due to an antagonistic interaction between the species. For the other co-cultures, an increase in metabolic activity and substrate assimilation was observed. The use of 2-strains co-cultures, i. e. *R. opacus* coupled with *M. hirsuta* or *M. parvus*, resulted in the maximum PHB yields (63 % w w^{-1} and 66.6 % w w^{-1}, respectively).

This work demonstrated the feasibility of methanotrophic co-cultures as a platform for improving the production of PHB from methane. However, further investigations are required to better understand the mechanisms of strain-to-strain specific interactions to tailor co-culture design.

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.

Acknowledgments

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