Journal Pre-proofs

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PII: S0048-9697(19)35128-9
DOI: https://doi.org/10.1016/j.scitotenv.2019.135136
Reference: STOTEN 135136

To appear in: *Science of the Total Environment*

Received Date: 20 August 2019
Revised Date: 21 October 2019
Accepted Date: 21 October 2019

Please cite this article as: Y. Rodríguez, P.I.M. Firmino, E. Arnáiz, R. Lebrero, R. Muñoz, Elucidating the influence of environmental factors on biogas-based polyhydroxybutyrate production by *Methylocystis hirsuta* CSC1, *Science of the Total Environment* (2019), doi: https://doi.org/10.1016/j.scitotenv.2019.135136

Elucidating the influence of environmental factors on biogas-based polyhydroxybutyrate production by *Methylocystis hirsuta* CSC1

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Abstract

The valorization of biogas as a feedstock for the generation of added-value bioproducts will play a key role on the sustainability of anaerobic digestion. The present work assessed the influence of key environmental parameters (O_2:CH_4 ratio, temperature and nitrogen source) on the growth and polyhydroxybutyrate (PHB) synthesis under nitrogen limiting conditions of the type II methanotroph *Methylocystis hirsuta* CSC1 using biogas as a feedstock. The O_2:CH_4 ratios tested (1:1, 1.5:1 and 2:1) did not affect significantly *M. hirsuta* CSC1 growth yields (~5 g TSS mol^{-1} CH_4), although lower CH_4 removal rates were reached under O_2-

limiting conditions (ratio 1:1). The highest PHB content (45 wt%) was achieved at a ratio 2:1 and was threefold higher than those obtained at lower ratios (~15 wt%). The increase in temperature from 15 to 25ºC resulted in increases in the growth yield (from 5 to 6 g TSS mol^{-1} CH_4) and PHB content (from 32 to 40 wt%). Conversely, the lowest PHB content (30 wt%) was reached at 37ºC, together with a negligible growth under nutrient sufficient conditions. The nitrogen source also played a key role on both *M. hirsuta* CSC1 growth and PHB synthesis. Thus, ammonium resulted in the highest growth yield (7 g TSS mol^{-1} CH_4), although the maximum PHB content was achieved when biomass was previously grown in nitrate as the nitrogen source (41 wt%). Nitrite exerted an inhibitory effect on *M. hirsuta* CSC1 growth.

Keywords: biogas valorization, methane conversion, methanotrophs, *Methylocystis hirsuta*, microbial cell factories, polyhydroxybutyrate production

1. Introduction

Polyhydroxyalkanoates (PHAs) are microbial plastics that are naturally produced under nutrient limitation and carbon surplus by over 300 bacterial strains (Pieja et al., 2017; Strong et al., 2016). These biodegradable and biocompatible polyesters are regarded as promising candidates to substitute petroleum-derived plastics owing to their similarity to various conventional thermoplastics and elastomers (Choi & Lee, 1999). Approximately 24 companies are nowadays involved in PHA manufacturing, Metabolix (USA), Meredian (USA), Bio-on (Italy) and Tianjin Green Bioscience (China) corporations being at the forefront of poly-3-hydroxybutyrate (PHB) and poly(3-hydroxybutyrate-*co*-3-

hydroxyvalerate) (PHBV) production worldwide (Chen, 2009; Singh et al., 2017). Despite the environmental advantages of bioplastics, the development of the PHA market (expected to reach up to $250 \cdot 10^3$ t $year^{-1}$ by 2021) is still hampered by their high costs of production and purification, which are currently 5-10 times higher than those associated with conventional plastics (nova-Institut, 2016; Raza et al., 2018). Research efforts are focused on reducing the operating costs associated with downstream PHA processing and the acquisition of the carbon source (the latter accounting for 30 – 40% of the total cost) (Cantera et al., 2018; Lee & Na, 2013) .

In this context, biogas has emerged as a potential low-cost substrate for PHA production due to its high content of methane (50 - 70%) (López et al., 2018). Biogas from the anaerobic microbial conversion of organic matter (i.e. agro-industrial residues, urban solid waste or sewage sludge) has been typically used for heat and power generation, upgraded into biomethane for grid injection, or simply flared. In the last decade, the number of biogas production facilities in Europe has increased by 186%, from 6227 in 2009 to 17783 units and a total installed electric capacity of 10532 MW by the end of 2017 (EBA, 2019).

Methane-oxidizing bacteria (MOB), also known as methanotrophs, are strict aerobic microorganisms that use methane as their sole carbon and energy source. Unlike other methanotrophs, type II MOB utilize the serine pathway for formaldehyde assimilation and possess the ability to produce PHAs from dilute methane emissions or biogas under nutrient-limited (usually nitrogen) conditions (Hanson & Hanson, 1996; Lindner et al., 2007). Among the PHA-synthesizing methanotrophs, *Methylocystis*, *Methylosinus* and *Methylocella* are the most important genera, with PHA contents ranging from 20 to 60 wt% (Bordel et al., 2019a; Cantera et al., 2019). In particular, the strain *Methylocystis hirsuta* CSC1, isolated for the first time from an aquifer in CA (USA) in the mid-eighties (Lindner et al., 2007), exhibits a high PHA-accumulating capacity, with PHA contents of 43 and 52 wt% using biogas and natural

gas (90% methane) as feedstock, respectively, and a high metabolic plasticity (López et al., 2018; Rahnama et al., 2012). PHA synthesis in methanotrophs depends on environmental factors such as pH, temperature, and the concentrations of methane, oxygen, carbon dioxide, macronutrients (nitrogen, phosphorous, etc.) and micronutrients (copper, cobalt, etc.) (Karthikeyan et al., 2015; Strong et al., 2016; Zhang et al., 2017). Unfortunately, there is still a limited understanding of the influence of most of these environmental factors on PHA accumulation by type II methanotrophs. In this regard, Rostkowski et al., (2013) reported that the oxygen requirements and the effect of the nitrogen source on the kinetics and stoichiometry of growth and PHB accumulation of type-II methanotrophs are highly organism-specific. Nitrate and ammonia, even gaseous N_2, can be assimilated by obligate methanotrophs (Murrell and Dalton, 1983). Nitrate and ammonium concentrations of up to 40 mM can support methane oxidation by the type-II MOB R-45379 (Hoefman et al., 2014). Despite the versatile nitrogen metabolism of methanotrophs, there is a limited number of studies about the nitrite detoxification ability of certain type-II microorganisms. This harmful compound can be produced either from nitrate reduction or from ammonia oxidation via hydroxylamine by some type-II methanotrophs (Bowman et al., 1993; Hoefman et al., 2014). On the other hand, the optimum growth temperature for most methanotrophic bacteria typically ranges from 25 to 35ºC (Hanson and Hanson, 1996), although some strains have been isolated from psychrophilic and thermophilic environments (Dunfield, 2009). Indeed, *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP were able to grow and accumulate PHB at 37ºC (Lindner et al., 2007).

This work aimed at assessing the influence of key environmental parameters such as the O_2:CH_4 ratio, the type of nitrogen source (NO_3^-, NO_2^- and NH_4^+) and the temperature on the growth and PHB synthesis of the type II methanotroph *Methylocystis hirsuta* CSC1 using biogas as the sole carbon and energy source.

2. Materials and methods

2.1. Microorganism and chemicals

The methanotrophic strain *Methylocystis hirsuta* CSC1, purchased from Leibniz-Institut DSMZ (DSM no. 18500), was inoculated (10% v/v) under sterile conditions in 125-mL crimp-sealed serum bottles containing 50 mL of nitrate mineral salt (NMS) medium. NMS medium (pH of 6.8) was composed of (g L^{-1}): 1.0 KNO_3, 1.1 $MgSO_4 \cdot 7H_2O$, 0.8 $Na_2HPO_4 \cdot 12H_2O$, 0.26 KH_2PO_4 and 0.2 $CaCl_2 \cdot 2H_2O$; and 1 mL of trace element solution (g L^{-1}): 0.3 $Na_2MoO_4 \cdot 2H_2O$, 0.3 $Na_2EDTA \cdot 2H_2O$, 1 $CuSO_4 \cdot 5H_2O$, 0.5 $FeSO_4 \cdot 7H_2O$, 0.4 $ZnSO_4 \cdot 7H_2O$, 0.03 $CoCl_2$, 0.02 $MnCl_2 \cdot 4H_2O$, 0.015 H_3BO_3, 0.01 $NiCl_2 \cdot 6H_2O$ and 0.38 Fe-EDTA. Subsequently, the bottles headspace (75 mL) was flushed under sterile conditions for 5 minutes with filtered oxygen (0.22 μm; Millex GP, Merck). Then, 25 mL of the oxygen headspace atmosphere were replaced by methane, which resulted in an O_2:CH_4 concentration ratio of 66.7:33.3% (v/v). The cultures were incubated at 30°C and 200 rpm in an orbital shaker (MaxQ 4000; Thermo Scientific, USA) for ~7 days. The headspace atmosphere of the bottles was replaced 5 times upon CH_4 depletion. Unless otherwise specified, this inoculum was prepared prior the start-up of i) the test series assessing the influence of environmental conditions on *M. hirsuta* CSC1 growth and ii) the growth of *M. hirsuta* CSC1 biomass for the tests series assessing the influence of the environmental conditions on PHA accumulation.

Gas cylinders of CH_4 (purity ≥ 99.995%), O_2 (≥ 99.5%), He (≥ 99.5%) and synthetic biogas (70% CH_4, 30% CO_2) were purchased from Abelló Linde S.A. (Barcelona, Spain). Potassium nitrate was obtained from Cofarcas S.A. (Burgos, Spain), whereas the rest of the salts required for the preparation of the mineral medium were acquired from PanReac AppliChem (Barcelona, Spain). Commercial PHBV (with a PHV content of 12% mol) for the

preparation of standard biopolymer solutions in chloroform was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Experimental procedure

All tests described below were performed batchwise in duplicate in 2.2-L serum bottles (working volume of 0.4 L) capped with butyl-rubber stoppers and aluminum screw caps (Fig. 1). Unless otherwise specified, bottles were incubated under mesophilic conditions (25 °C) with an initial pH of 7.1 in a thermostated room and magnetically stirred at 300 rpm (Poly 15 Variomag, Thermo Fisher Scientific).

The CH_4, CO_2 and O_2 headspace concentrations were periodically monitored by gas chromatography coupled with a thermal conductivity detector (GC-TCD) in all tests. The characterization of the culture broth was carried out by withdrawing 4 mL-samples and determining biomass concentration via optical density measurement and PHB content via gas chromatography-mass spectrometry (GC-MSD). The latter was carried out exclusively during the accumulation assays. pH was recorded at the beginning and end of each test. Additionally, initial and final concentrations of total nitrogen (TN), $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ were analyzed in the Test series 3-G (see section 2.2.5).

2.2.1. Test 1-G: Influence of the O_2:CH_4 ratio on M. hirsuta *CSC1 growth*

Three different headspace compositions (CH_4:O_2:CO_2:He) were tested in order to assess the influence of the O_2:CH_4 ratio on *M. hirsuta* CSC1 growth using biogas as a feedstock: 29.2:29.2:12.5:29.2%, 29.2:43.8:12.5:14.6% and 29.2:58.3:12.5:0.0%, which corresponded to O_2:CH_4 molar ratios of 1:1, 1.5:1 and 2:1, respectively. These headspace compositions were prepared by supplying the corresponding volumes from synthetic biogas, O_2 and He cylinders into 25-L Tedlar gas sampling bags. Then, these gas mixtures were pumped into the corresponding bottles headspace by a gas compressor (C5, Electro AD,

Barcelona, Spain), ensuring a complete headspace replacement. Finally, the bottles were inoculated with fresh *M. hirsuta* CSC1 inoculum at 2.5% (v/v) and incubated over 2 weeks until CH_4 consumption ceased.

2.2.2. Test 1-A: Influence of the O_2:CH_4 ratio on PHB synthesis by M. hirsuta *CSC1*

The capacity of *M. hirsuta* CSC1 to synthesize PHB was assessed at O_2:CH_4 molar ratios of 1:1, 1.5:1 and 2:1. First, *M. hirsuta* CSC1 was grown at 25°C and 300 rpm in six 2.2-L serum bottles containing 400 mL of NMS medium under an O_2:CH_4 atmosphere of 66.7:33.3% until complete methane depletion (~10 days). Then, the biomass was harvested from the culture broth by centrifugation at 10000 rpm for 8 min (Sorvall Legend RT+; Thermo Scientific, USA) and re-suspended into 220 mL of nitrate-free mineral salt (NFMS) medium (prepared based on the NMS receipt described above without nitrate addition). An aliquot of 30 mL of this concentrated culture was inoculated (7.5% v/v) in each bottle resulting in an initial biomass concentration (estimated as total suspended solids, TSS) of 253.5 mg L^{-1}. Finally, the air headspace of the bottles was replaced with gas mixtures (prepared as above described) containing O_2:CH_4 molar ratios of 1:1, 1.5:1 and 2:1, and incubated until CH_4 consumption ceased (~3 weeks).

2.2.3. Test 2-G: Influence of the temperature on M. hirsuta *CSC1 growth*

M. hirsuta CSC1 growth on biogas was assessed at different temperatures (15, 25 and 37°C), which were controlled using either a thermostatic water bath (15 °C) or temperature-controlled rooms (25 and 37°C). The bottles were provided with an initial headspace CH_4:O_2:CO_2 atmosphere of 29.2:58.3:12.5% (O_2:CH_4 ratio of 2:1), inoculated with fresh *M. hirsuta* CSC1 inoculum at 2.5% (v/v) (initial TSS of 14 mg L^{-1}) and incubated until CH_4 consumption ceased (55, 16 and 30 days for 15, 25 and 37 °C, respectively).

2.2.4. Test 2-A: Influence of the temperature on PHB synthesis by M. hirsuta *CSC1*

The capacity of *M. hirsuta* CSC1 to synthesize PHB under nitrogen deprivation was assessed at 15, 25 and 37°C. First, *M. hirsuta* CSC1 biomass was grown at 25°C under an O_2:CH_4 headspace composition of 66.7:33.3% and harvested as previously described in section 2.2.2. The bottles in Test 2-A were initially provided with a headspace CH_4:O_2:CO_2 composition of 29.2:58.3:12.5% (O_2:CH_4 ratio of 2:1), inoculated with the harvested biomass at 7.5% (v/v) (initial TSS of 269.3 mg L^{-1}) and incubated at the target temperatures until CH_4 consumption ceased (~3 weeks).

2.2.5. Test 3-G: Influence of the nitrogen source on M. hirsuta *CSC1 growth*

The influence of the nitrogen source (NH_4Cl, $NaNO_3$ and $NaNO_2$) on *M. hirsuta* CSC1 growth on biogas was assessed by modifying the composition of the NMS medium accordingly, while maintaining a TN concentration of 138 mg N L^{-1}. The bottles (pH = 7.0 ± 0.1) were initially provided with a headspace CH_4:O_2:CO_2 composition of 29.2:58.3:12.5% (O_2:CH_4 ratio of 2:1), inoculated with fresh *M. hirsuta* CSC1 inoculum at 2.5% (v/v) (initial TSS of 21.3 mg L^{-1}) and incubated until CH_4 consumption ceased (~ 2 weeks).

2.2.6. Test 3-A: Influence of the nitrogen source during M. hirsuta *CSC1 growth on PHB synthesis*

The influence of the nitrogen source (NH_4Cl, $NaNO_3$ and $NaNO_2$) during the growth phase on the capacity of *M. hirsuta* CSC1 to synthesize PHB was evaluated. Thus, the biomass grown in Test 3-G was harvested separately by centrifugation (at 10000 rpm for 8 min and re-suspended into 70 mL of NFMS per bottle). Then, the bottles were provided with an initial headspace CH_4:O_2:CO_2 composition of 29.2:58.3:12.5% (O_2:CH_4 ratio of 2:1), inoculated with the nitrogen-free cell suspension at 7.5% (v/v) (initial TSS of 192 ± 13 mg L^{-1}) and incubated until CH_4 consumption ceased (~ 3 weeks).

2.3. Analytical methods

The gas concentrations of CH_4, O_2 and CO_2 were periodically monitored by using a Bruker 430 GC-TCD (Bruker Corporation, Palo Alto, USA) equipped with CP-PoraBOND Q and CP-Molsieve 5A columns as described by Estrada et al. (2014). pH and TSS in the culture broth were analyzed according to APHA (2015), whereas the optical density of the culture samples was determined at 600 nm (OD_{600}) by spectrophotometry (UV-2550; Shimadzu, Japan). NO_2^- and NO_3^- concentrations were quantified at the beginning and at the end of Test 3-G by high performance liquid chromatography-ion conductivity (HPLC-IC) according to Posadas et al. (2013). TN concentration was analyzed by chemiluminiscense using a TOC-V analyzer equipped with a TNM-1 unit (Shimadzu, Japan).

PHB extraction from *M. hirsuta* CSC1 biomass was conducted by modifying the method described by Zúñiga et al. (2011), which is based on the hydrolysis and propanolysis of the PHB. Two liquid samples (1.5 mL each) were centrifuged at 10000 rpm for 10 min. After discarding supernatant, 1 mL of propanol containing hydrochloric acid (80:20% (v/v)) and 2 mL of trichloromethane were added to the biomass pellets. Benzoic acid in propanol (10 µL) was used as internal standard solution (5 g L^{-1}). Then, samples were agitated by vortexing and incubated for 4h at 100ºC in a thermoreactor. After cooling down to room temperature, 1 mL of deionized water was added to the samples, which were agitated again. The organic phase was collected after phase separation and filtered (0.22 µm; Merck). The quantitative determination of PHB was carried out using an Agilent 7820A gas chromatograph (GC) coupled with a 5977E mass spectrometer detector (MSD) (Agilent Technologies, USA) and equipped with a DB-WAX column (Agilent Technologies, USA). The GC-MSD temperature program for PHB determination can be found elsewhere (López et al. 2014).

3. Results and discussion

3.1 Influence of the O_2:CH_4 ratio on M. hirsuta *CSC1 growth*

At the O_2:CH_4 ratios of 1.5:1 and 2:1, CH_4 (provided at an initial concentration of 161 ± 2 g m^{-3}) was fully depleted within the first 10 days (Fig. 2a). However, at the O_2:CH_4 ratio of 1:1, only 70% of the CH_4 initially present in the headspace was consumed as a result of O_2 limitation (Fig. 2a). In fact, methanotrophs require approximately 1.5 to 2 mol O_2 to oxidize 1 mol CH_4 (Karthikeyan et al., 2015). Therefore, biomass production (195 ± 6 mg TSS L^{-1}) at the lowest O_2:CH_4 ratio was approximately 23% lower than that obtained at O_2:CH_4 ratios of 1.5 and 2 (252 ± 4 mg TSS L^{-1}) (Fig. 2b). Nevertheless, comparable growth yields (Y_{X/CH_4}) were obtained at all O_2:CH_4 ratios tested (4.9 ± 0.2, 5.0 ± 0.1 and 5.2 ± 0.1 g TSS mol^{-1} CH_4 at ratios of 1:1, 1.5:1 and 2:1, respectively). Similarly, Rostkowski et al. (2013) also observed no significant difference in the growth yield of two type II methanotrophs – *Methylosinus trichosporium* OB3b (0.66 ± 0.03 g VSS g^{-1} CH_4) and *Methylocystis parvus* OBBP (0.55 ± 0.03 g VSS g^{-1} CH_4) – at different O_2:CH_4 ratios (from 0.37:1 to 1.47:1) with nitrate as the nitrogen source.

The O_2:CH_4 consumption molar ratios were similar at all tested conditions (1.4 ± 0.1, 1.5 ± 0.1 and 1.5 ± 0.0 mol O_2 mol^{-1} CH_4 at ratios of 1:1, 1.5:1 and 2:1, respectively). These values were close to the theoretical consumption molar ratio for type II methanotrophs growth (1.5 mol O_2 mol^{-1} CH_4) (Asenjo & Suk, 1986). Additionally, these results were in accordance with previous studies conducted with *M. trichosporium* OB3b, *M. parvus* OBBP and *M. hirsuta* CSC1, where O_2:CH_4 consumption molar ratios ranging from 1.3 to 1.5 mol O_2 mol^{-1} CH_4 were observed (López et al., 2018; Rostkowski et al., 2013; Zhang et al., 2017). Finally, it should be highlighted that O_2 availability severely influenced the maximum CH_4 removal rates (RRs). Thus, *M. hirsuta* CSC1 growth at an O_2:CH_4 ratio of 1:1 resulted in lower RR (26.5 ± 0.8 g CH_4 m^{-3} d^{-1}) than the cultivation at O_2:CH_4 ratios of 1.5 and 2 (34.0 ±

1.5 and 36.8 ± 0.7 g CH_4 m^{-3} d^{-1}, respectively). In this context, since the Henry´s law constants of O_2 and CH_4 (H^{cp} = $1.4 \cdot 10^{-5}$ for methane and H^{cp} = $1.3 \cdot 10^{-5}$ mol m^{-3} Pa^{-1} for oxygen at 298.15 K (Sander, 2015)) are similar, low initial concentrations of O_2 in the gas phase entail low O_2 gas-liquid mass transfer rates, which can ultimately mediate O_2 limitation in the culture broth and reduced CH_4 RR.

3.2 Influence of the O_2:CH_4 ratio on PHB synthesis by M. hirsuta *CSC1*

Under N-limiting conditions, O_2 and CH_4 consumption by *M. hirsuta* CSC1 exhibited the same pattern as in the growth assay at all tested O_2:CH_4 ratios (Fig. 2c). However, the O_2:CH_4 consumption molar ratios observed during PHB synthesis were higher than those recorded in the growth test (1.7 ± 0.0, 1.7 ± 0.1 and 1.6 ± 0.1 mol O_2 mol CH_4^{-1} at 1:1, 1.5:1 and 2:1 ratios, respectively) and also exceeded the theoretical value for PHB accumulation by type II methanotrophs (1.5 mol O_2 mol^{-1} CH_4) (Asenjo & Suk, 1986). Previous studies with *M. trichosporium* OB3b, *M. parvus* OBBP and *M. hirsuta* CSC1 reported a decrease in the O_2:CH_4 consumption molar ratio under N-limiting conditions due to the electron deviation to PHB accumulation (López et al., 2018; Rostkowski et al., 2013). The maximum CH_4 RRs recorded in the assays carried out at 1:1, 1.5:1 and 2:1 O_2:CH_4 ratios were 22.9 ± 1.7, 28.4 ± 0.3 and 36.1 ± 1.7 g CH_4 m^{-3} d^{-1}, respectively.

M. hirsuta CSC1 reached the maximum PHB content within 5 and 2 days at the O_2:CH_4 ratios 1:1 and 1.5:1, while 8 days were needed at the ratio 2:1 (Fig. 2d). However, the latter condition resulted in a maximum PHB content threefold higher (45.3 ± 1.7 wt%) than those obtained at 1:1 and 1:5 ratios (16.1 ± 0.2 and 15.0 ± 1.8 wt%, respectively) (Fig. 2d). This corresponded to PHB yields (Y_{PHB/CH_4}) of 2.3 ± 0.0, 2.6 ± 0.1 and 6.9 ± 0.1 g PHB mol^{-1} CH_4 at 1:1, 1.5:1 and 2:1 ratios, respectively. At O_2:CH_4 ratios of 1:1 and 1.5:1, the analysis of the carbon mass balance (data not shown) suggests that carbon fluxes support a major

mineralization rather than PHB synthesis through the serine cycle, i.e.: CH_4 is preferentially used for energy generation rather than PHB synthesis as a result of the metabolic stress caused by O_2 limitation in the assays. The results obtained at the O_2:CH_4 ratio of 2:1 were in agreement with those reported by López et al. (2018), who found a maximum PHB content of 45 ± 1 wt% (Y_{PHB/CH_4} = 7.0 ± 0.0g PHB mol^{-1} CH_4) when using biogas as a feedstock for biopolymer production by *M. hirsuta* CSC1 in batch assays at a similar O_2:CH_4 ratio (1.75:1). According to Karthikeyan et al. (2015), O_2 requirements during biopolymer synthesis depend on the methanotroph strain, and limiting O_2 concentrations can negatively affect PHB accumulation. Therefore, an O_2:CH_4 ratio higher than 1.5:1 is required to maximize PHB synthesis in *M. hirsuta* CSC1.

3.3 Influence of the temperature on M. hirsuta *CSC1 growth*

The growth of *M. hirsuta* CSC1 at three different temperatures (15, 25 and 37°C) was evaluated (Fig. 3a and 3b). CH_4 removals > 95% were achieved within 10 and 43 days at 25 and 15°C, respectively. Neither *M. hirsuta* CSC1 growth nor CH_4 consumption was recorded at 37ºC for the 30 days of experiment. Despite its relevance, the influence of temperature on *M. hirsuta* CSC1 growth has been scarcely studied in literature. For instance, Lindner et al. (2007) pointed out that *M. hirsuta* CSC1 grows optimally at 30ºC and is not able to grow at 37ºC. On the contrary, other species from the genus *Methylocystis*, such as *M. parvus* (15-37ºC) and *M. rosea* (5-37ºC), possess the ability to grow at 37ºC, although optimal values are similar to those reported for *M. hirsuta* (28-30ºC and 27ºC for *M. parvus* and *M. rosea*, respectively) (Tsyrenzhapova et al., 2007).

The O_2:CH_4 consumption molar ratios were not temperature dependent, with values of 1.5 ± 0.1 and 1.6 ± 0.1 mol O_2 mol CH_4^{-1} at 15 and 25ºC, respectively, similar to the theoretical ones previously discussed in section 3.1.1. Conversely, cultures grown at 25ºC

exhibited a maximum RR of 36.5 ± 0.2 g CH_4 m^{-3} d^{-1} during the exponential phase, whereas, at 15ºC, *M. hirsuta* CSC1 supported a maximum RR of 6.2 ± 0.2 g CH_4 m^{-3} d^{-1} after a very long lag phase (~ 22 d). Thus, the kinetics of methane biodegradation by *M. hirsuta* CSC1 exhibited a great sensitivity to temperature. Finally, maximum biomass concentrations of 198.5 ± 12.6 and 244.2 ± 3.8 mg TSS L^{-1} were recorded at 15 and 25ºC, respectively, which resulted in biomass yield coefficients (Y_{X/CH_4}) of 5.0 ± 0.2 and 5.8 ± 0.2 g TSS mol^{-1} CH_4, respectively.

3.4 Influence of the temperature on PHB synthesis by M. hirsuta *CSC1*

The temperature of cultivation did influence not only the extent of CH_4 oxidation, but also PHB synthesis in the absence of nitrogen (Fig. 3c and 3d). Methane conversion reached 60% within the first 15 days at 15ºC, while a nearly complete methane conversion (>90%) was achieved at 25 and 37ºC within 15 days, with maximum RRs of 38.8 ± 0.8 and 36.4 ± 2.8 g CH_4 m^{-3} d^{-1}, respectively. At 15ºC, the maximum CH_4 RR was approximately half of the RRs values recorded at 25 and 37ºC (17.7 ± 0.2 g CH_4 m^{-3} d^{-1}). No significant influence of the temperature on the O_2:CH_4 consumption molar ratios was observed (1.5 ± 0.1, 1.6 ± 0.0 and 1.7± 0.2 mol O_2 mol CH_4^{-1} at 15, 25 and 37ºC, respectively).

On the other hand, the highest PHB content in *M. hirsuta* CSC1 was obtained at 25ºC with a PHB accumulation of 39.7 ± 0.2 wt%, followed by a 32.0 ± 0.1 wt% at 15ºC and 30.1 ± 0.5 wt% at 37ºC. This similar methane consumption at different PHB contents at 25 and 37ºC suggests that temperature exerts a significant impact on PHB yield. In this context, similar PHB yields of 6.6 ± 0.0 and 6.9 ± 0.3 g PHB mol^{-1} CH_4 were observed at 15 and 25ºC, respectively, whereas the PHB yield at 37ºC was 5.0 ± 0.5 g PHB mol^{-1} CH_4. Despite its sensitivity to temperature, *M. hirsuta* CSC1 exhibited a high PHB synthesis capacity within the range of temperatures tested. It should be stressed that PHB accumulation tests at different temperatures were carried out with biomass pre-cultivated at 25ºC. Interestingly, no

lag phase was observed during the accumulation stage at any of the temperatures tested. Moreover, the 10 day pre-cultivation phase was unlikely to induce any temperature adaptation based on the fact that experiments were carried out with an axenic strain.

The PHB contents herein recorded were in good agreement with previous findings for *M. hirsuta* (42.5% at 30ºC (Rahnama et al., 2012) or 34.6% at 25ºC (García-Pérez et al., 2018)). In a recent study, Pérez et al. (2019) evaluated the PHB accumulation capacity of mixed cultures enriched from *Sphagnum* and *Sphagnum* + activated sludge at 25, 30 and 37ºC. Enrichments from *Sphagnum* at 25ºC resulted in the highest PHB contents (~18 wt%), which were mediated by the dominance of the *Methylocystis* genus (up to 46%) in the community. Interestingly, while *M. hirsuta* CSC1 in this study was not able to grow at 37ºC (likely due to the heat stress that did not enable DNA replication in cells and inhibited the activity of the base excision repair system) (Kantidze et al., 2016), this microorganism was able to synthetize PHB at such a high temperature. These outcomes are hardly comparable with literature since, to the best of the author's knowledge, no previous studies have addressed the PHB synthesis capacity of pure methanotrophic cultures at different temperatures.

3.5 Influence of the nitrogen source on M. hirsuta *CSC1 growth*

Methane conversions of 80% and 100%, along with maximum RRs of 31.4 ± 1.3 and 35.6 ± 1.9 g CH_4 m^{-3} d^{-1}, were recorded when ammonium and nitrate, respectively, were used as the sole nitrogen source (Fig. 4a and 4b). The lower rate of methane oxidation in the presence of ammonium at 10 mM NH_4^+ evidenced a slight competitive inhibition between methane and ammonium for the enzyme particulate methane monooxygenase (pMMO) (Bordel et al., 2019a). In this regard, previous studies reported greater inhibitory effects on methane biodegradation at 5 mM ammonium on some type II MOB strains, such as

Methylocystis sp. and *Methylosinus sporium* (Nyerges & Stein, 2009). This suggests that *M. hirsuta* CSC1 exhibits a significantly greater tolerance than its close relative species. Interestingly, a negligible growth was observed in the presence of nitrite (10 mM NO_2^-), which highlights the inhibitory effect of nitrite on *M. hirsuta* metabolism, more specifically on the formate dehydrogenase activity (Jollie & Lipscomb, 1990). Unfortunately, the mechanisms underlying this inhibition have not been yet elucidated (King and Schnell, 1994).

Nevertheless, the genome sequence of the *M. hirsuta* CSC1, which has recently been published, revealed that this strain possesses genes involved in denitrification, which would allow nitrite reduction to NO and further to N_2O (Bordel et al., 2019a). Thus, despite its detrimental effect on CH_4 oxidation, a certain tolerance was observed in previous works investigating the toxic effect of nitrite on mixed methanotrophic communities (Dunfield & Knowles, 1995) and pure cultures (NMS amended with nitrite (2mM) in *Methylocystis* sp.) (Nyerges & Stein, 2009).

M. hirsuta CSC1 cultures grown in nitrate supported greater biomass concentrations (TSS = 264.4 ± 0.7 mg L^{-1}) than those grown in ammonium (TSS = 239.3 ± 2.9 mg L^{-1}) as a result of the incomplete methane oxidation in the presence of the latter. Similarly, the biomass yield depended on the nitrogen sources (Y_{X/CH_4} = 7.10 ± 0.10 and 5.80 ± 0.01 g TSS mol^{-1} CH_4 were estimated for ammonium and nitrate, respectively). Although biomass growth rates were quite similar using both media, ammonium seems to be the preferred N source for *M. hirsuta* CSC1 in terms of biomass yield. However, the influence of the nitrogen source on the metabolism of methanotrophs in strain dependent. For instance, ammonium mediated greater biomass yields than nitrate with concomitant lower growth rates for the type-II methanotrophs *Methylocystis* sp. Rockwell and *Methylocystis* sp. WRRC1 (Tays et al., 2018), whereas nitrate was the preferred nitrogen source in terms of biomass productivity for *M. trichosporium* OB3b (Rostkowski et al., 2013; Tays et al., 2018). Moreover, the results

obtained in the present work were in agreement with Bordel et al. (2019b), who predicted (using a Genome Scale Metabolic Model for *M. hirsuta*) higher biomass yields when using ammonium owing to the fact that no reducing power is needed for the conversion of NH_4^+ to organic nitrogen. The final pH of the culture media was also affected by the nitrogen source, with a final pH value of 4.5 when using ammonium, and pH values of 6.3 and 6.2 in the presence of nitrate or nitrite, respectively. This decrease in pH during CH_4 biodegradation with NH_4^+ was likely due to the nitrification mediated by the enzyme pMMO. Indeed, when ammonium was used as a nitrogen source, an accumulation of nitrate up to 48.2 ± 8.0 mg L^{-1} occurred in the culture medium. In this sense, ammonium is initially oxidized by pMMO to hydroxylamine (NH_2OH) (Hanson & Hanson, 1996), which is a highly toxic intermediate further oxidized to nitrite (NO_2^-) due to the evolved ability of ammonia-oxidizing bacteria to encode the enzyme hydroxylamine oxidoreductase (HAO) (Stein & Klotz, 2011). Finally, nitrite is converted into NO_3^-.

3.6 Influence of the nitrogen source during M. hirsuta *CSC1 growth on PHB synthesis*

This test evaluated the PHB synthesis capacity of *M. hirsuta* CSC1 using biomass pre-grown in different nitrogen sources. The history and physiological status of the biomass influenced the capacity of the cells to oxidize CH_4 and accumulate PHB (Fig. 4c and 4d). Cultures previously grown in ammonium converted 21.9 ± 0.6% of the initial methane supplied, whereas those grown in nitrate achieved a total conversion of 62.8 ± 0.0% within 13 days of experiment (Fig. 4c). Similarly, nearly a threefold decrease in the methane removal rate was observed in cultures previously grown in ammonium (13.3 ± 0.9 g CH_4 m^{-3} d^{-1}) compared with cultures previously grown in nitrate (38.3 ± 2.4 g CH_4 m^{-3} d^{-1}). Similarly, the PHB content of cultures grown in nitrate accounted for 41.1 ± 0.1%, while a PHB content of 18.0 ± 0.7% was observed in biomass grown in ammonium (Fig. 4d). These results are in agreement with those of Rostkowski et al. (2013) who reported higher PHB contents under N

deprivation when *M. trichosporium* OB3b was previously grown in nitrate than in ammonium (29 versus 12%, respectively) at an O_2:CH_4 ratio of 1:1. However, the PHB contents achieved with species from the same genus (*M. parvus* OBBP) do not seem to support an overall pattern (60 wt% under ammonium limitation and 14 wt% under nitrate limitation).

The highest experimental yield in the present study was recorded under nitrate deprivation (Y_{PHB/CH_4} = 7.3 ± 0.1 g PHB mol^{-1} CH_4), which was close to the theoretical value (8.6 g mol^{-1}) estimated by Yamane (1993) and to other experimental values reported in literature (7.0 – 8.8 g PHB mol^{-1} CH_4 for *M. hirsuta* CSC1 and *Methylocystis sp.* GB 25, respectively) (López et al., 2018; Wendlandt et al., 2001). Under NH_4^+ limitation, the PHB yield herein obtained was substantially lower (Y_{PHB/CH_4} = 3.2 ± 0.1 g PHB mol^{-1} CH_4). The mechanism of influence on this counterproductive PHB may be ascribed to the acidic pH (4.5) of the culture broth at the end of the previous test, which negatively impacted the physiological status of *M. hirsuta* CSC1 (Test 3-G). According to Hanson and Hanson (1996), pH values ranging from 4.0 to 6.0 exert a detrimental effect on methane oxidation rates in methanotrophs. In order to uncouple the effects of the N source and pH on PHB synthesis, further investigation with pH control should be conducted in automated fermenters. Finally, the resulting O_2 demand for CH_4 conversion under nitrate deprivation correlated with the values reported in sections 3.1.2 and 3.1.4, under similar environmental conditions (~1.7 mol O_2 mol CH_4^{-1}).

4. Conclusions

The different O_2:CH_4 ratios tested did not significantly influenced *M. hirsuta* growth yields, although lower CH_4 RRs were achieved under O_2-limiting conditions. O_2 limitation also induced lower PHB contents under N deprivation. Higher growth yields and PHB accumulations were recorded when temperature increased from 15 to 25ºC. Interestingly,

while a negligible growth was observed at 37ºC, nitrogen limitation supported PHB contents up to 30 wt% at this temperature. Finally, ammonium resulted in higher growth yields compared to nitrate, while nitrite inhibited severely its growth. However, the PHB synthesis was higher for the culture previously grown in nitrate.

Acknowledgements

The authors acknowledge the financial support from the Spanish Ministry of Economy and Competitiveness (BES-2016-077160 contract and CTM2015-70442-R project), and the Regional Government of Castilla y León and the EU-FEDER (CLU 2017-09, VA281P18 and UIC 071). The authors are very grateful to J. Prieto and E. Marcos for their technical support.

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Figure captions

Fig. 1. Outline of the experimental setup of the tests carried out and main conditions assayed.

Fig. 2. Time course of CH_4 (squares), O_2 (circles), CO_2 (hexagons), TSS (inverted triangles) and PHB (triangles) concentrations during the growth test 1-G (a, b) and the accumulation test 1-A (c, d). Empty, half-filled and filled symbols correspond to the experiments carried out under the O_2:CH_4 atmospheres of 1:1, 1.5 and 2:1, respectively.

Fig 3. Time course of CH_4 (squares), O_2 (circles), CO_2 (hexagons), TSS (inverted triangles) and PHB (triangles) concentrations during the growth test 2-G (a, b) and the accumulation test 2-A (c, d). Empty, half-filled and filled symbols correspond to the experiments carried out at the temperatures of 15, 25 and 37°C, respectively.

Fig. 4. Time course of CH_4 (squares), O_2 (circles), CO_2 (hexagons), TSS (inverted triangles) and PHB (triangles) concentrations during the growth test 3-G (a, b) and the accumulation test 3-A (c, d). Empty, half-filled and filled symbols correspond to the experiments carried out with NH_4^+, NO_2^- and NO_3^- as nitrogen sources, respectively.

Fig. 1

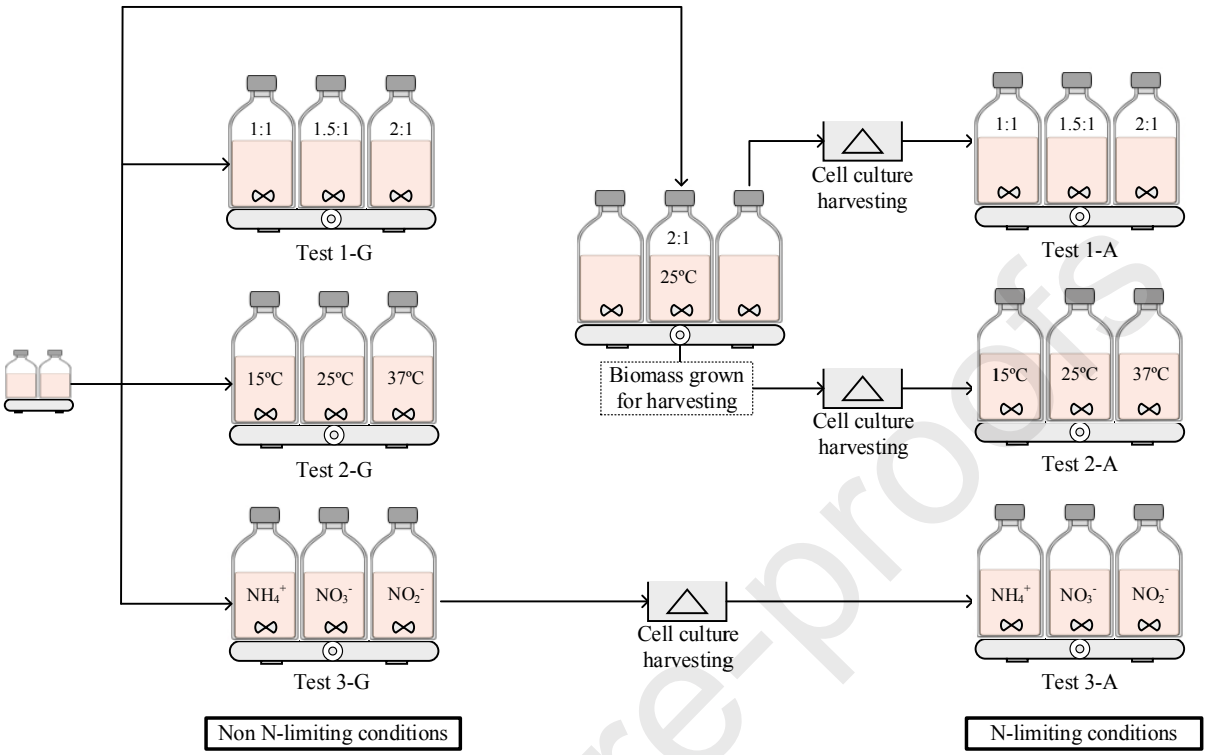


Fig. 2

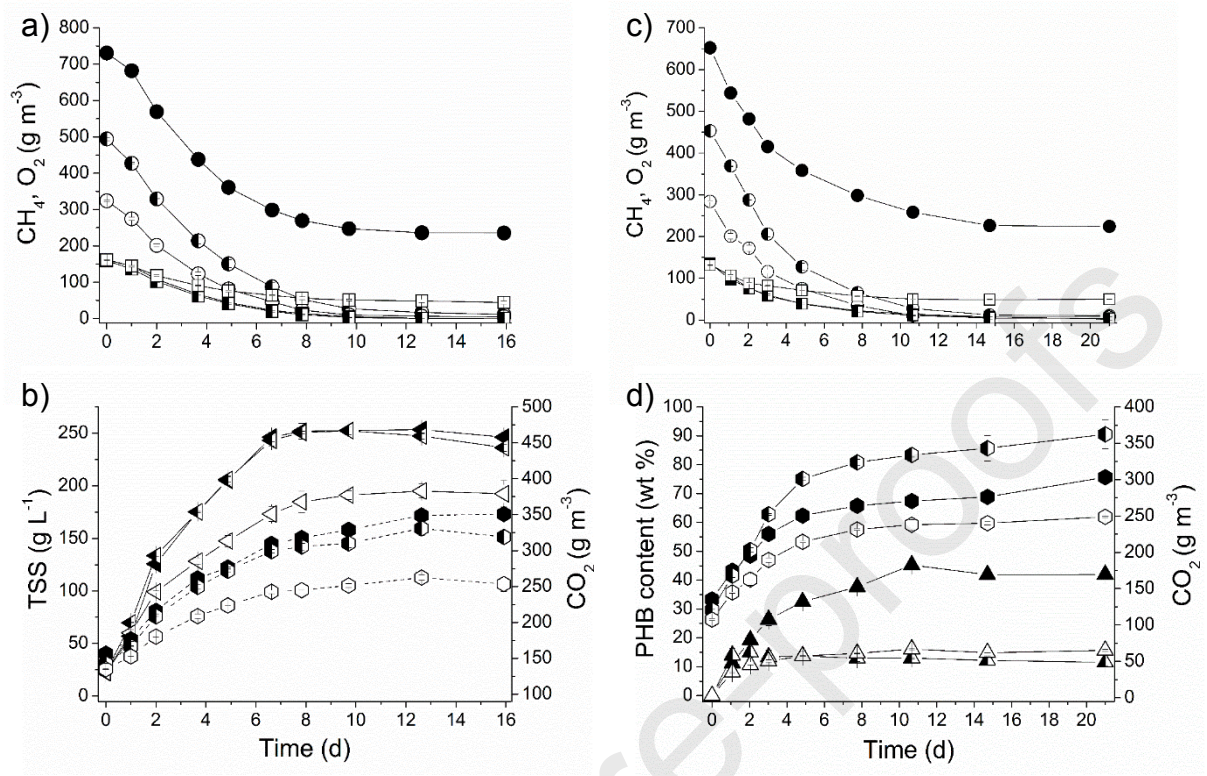


Fig. 3

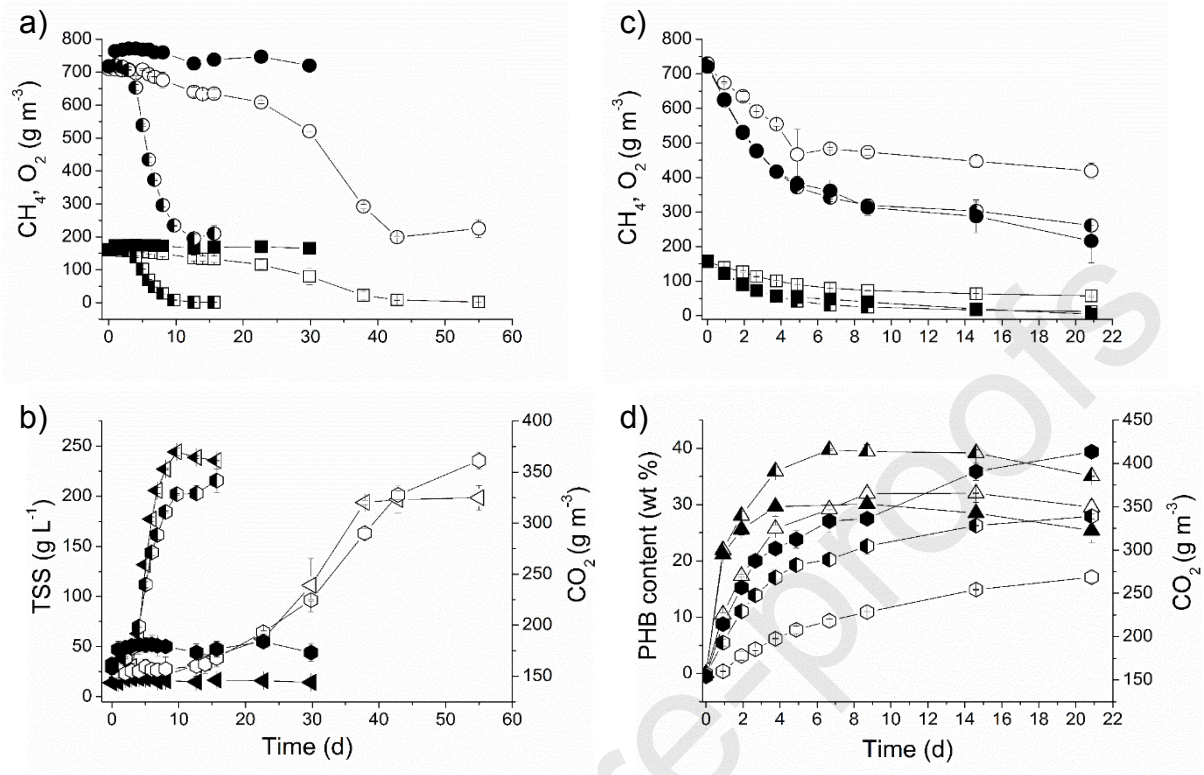
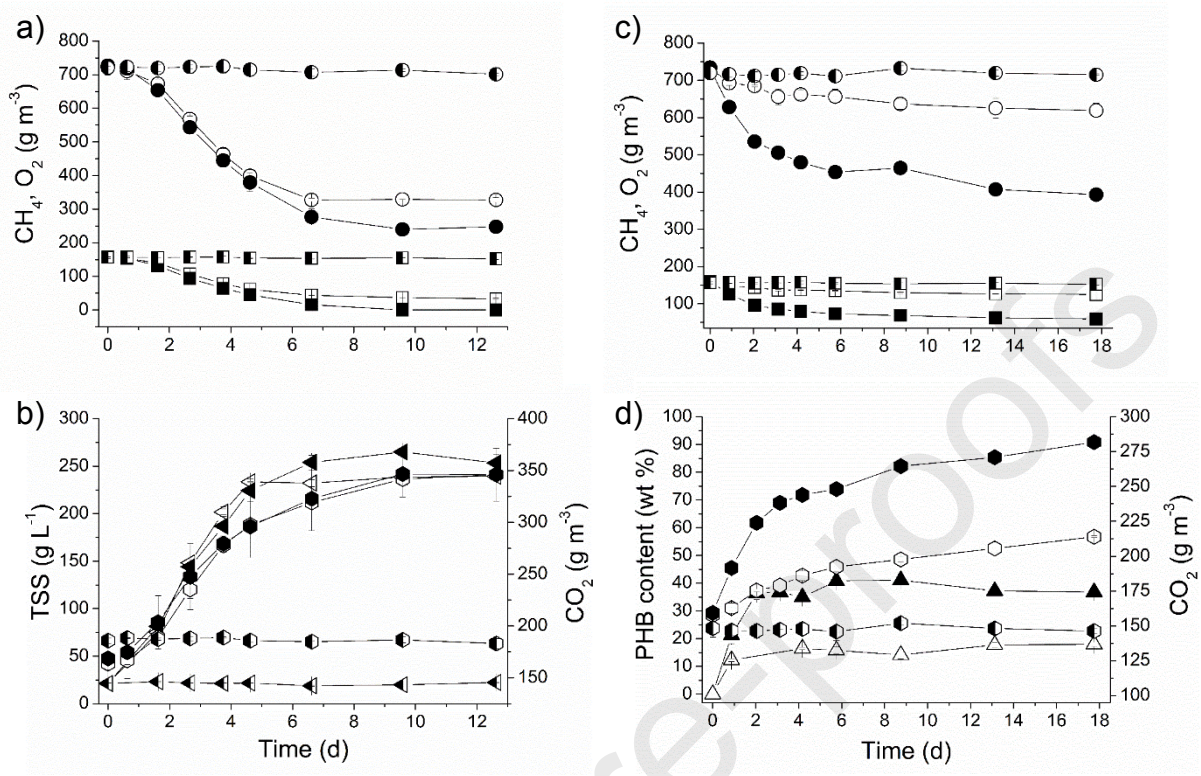


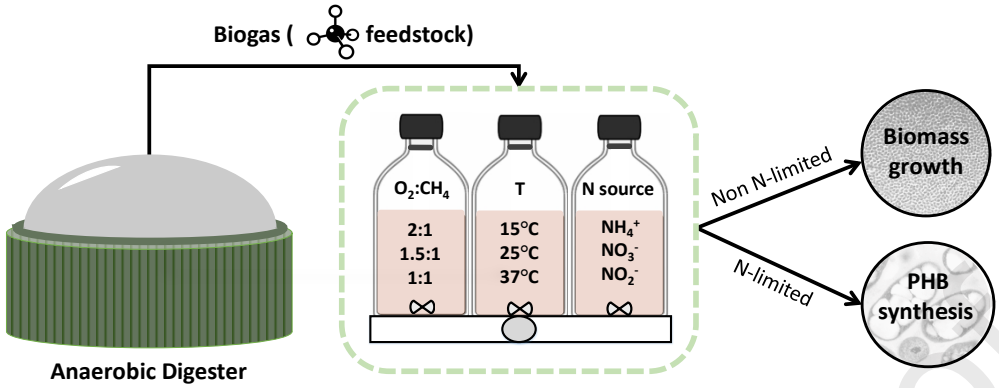
Fig. 4



Declaration of interests

☑ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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Yadira: Formal analysis, Investigation, Writing – original draft preparation

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Raquel: Conceptualization, Writing- Reviewing and Editing, Supervision, Funding Acquisition

Raúl: Conceptualization, Writing- Reviewing and Editing, Supervision, Funding Acquisition

Highlights

- *M. hirsuta* CSC1 was a suitable cell factory for PHB from biogas under N limitation
- The maximum PHB content (45 wt%) was reached at an O_2:CH_4 ratio of 2:1.
- PHB synthesis at 25ºC was higher than at 15 and 37 ºC.
- NO_3^- as N source supported a superior PHB accumulation compared to NH_4^+.