The effect of temperature during culture enrichment on methanotrophic polyhydroxyalkanoate production

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Abstract

Climate change and plastic pollution are likely the most relevant environmental problems of the 21st Century. Thus, one of the most promising solutions to remedy both environmental problems simultaneously is the bioconversion of greenhouse gases, such as methane (CH_4), into bioplastics (PHAs). However, the optimization of this bioconversion platform is still required to turn CH_4 biotransformation into a cost-effective and cost-competitive process. In this context, the research presented here aimed at elucidating the best temperature culture conditions to enhance both PHA accumulation and methane degradation. Six different enrichments were carried out at 25, 30 and 37ºC using different inocula and methane as the only energy and carbon source. CH_4 biodegradation rates, specific growth rates, PHA accumulations and the community structure were characterized. Higher temperatures (30 and 37ºC) increased the PHAs accumulation up to 30% regardless of the inoculum. Moreover, Methylocystis became the dominant genus (~ 30% of the total population) regardless of the temperature and inoculum used. This research demonstrated for the first time the fundamental role of temperature in increasing both the accumulation of PHAs and methane abatement during the enrichment of PHA cell-factories from methane, thus enhancing the cost-effectiveness of the process.

Keywords:

Bioplastics, Greenhouse Gas Abatement, Methanotrophs, PHA, methane.

1. Introduction

Nowadays, the two most important environmental issues that our society is facing are Climate Change and Plastic Pollution. In this sense, novel industrial strategies are necessary to abate toxic greenhouse gasses (GHG) and to substitute current used plastic materials without compromising present economic and industrial development.

Methane (CH_4) is currently the second most important GHG (Desai and Harvey, 2010) and its atmospheric concentration continues to rise at a yearly rate of 0.2 to 1% mainly due to anthropogenic activities (agriculture, livestock, waste management and energy production) which has caused growing interest, from a political and a scientific point of view, in greenhouse gas abatement ((EPA), 2017). On the other hand, the massive usage of plastic products and poor management of waste disposal has resulted in microplastic contamination of bodies of water, which injures wildlife through entanglement or ingestion of plastics and through the toxic and carcinogenic effect of some polymers. Additionally, it has been demonstrated that plastics can make their way up the food chain, causing similar health threats to humans. This situation has encouraged an intensive research to find and produce biodegradable and non-toxic plastics or biopolymers, such as polyhydroxyalkanoates (PHAs) which could definitively substitute and end the use of pollutant plastics (Löhr et al., 2017; Pieja et al., 2017; Sigler, 2014).

The PHAs are synthesized under nutrient-limited and carbon-excess conditions by different microorganisms (Pieja et al., 2017). The most common feedstock used for production of PHAs are glucose and fructose, but those carbon sources have a high price, due the market price of PHAs is higher (4-20 € $KgPHA^{-1}$) (Koller et al., 2017). PHAs are currently industrially produced by nearly thirty corporations (Cantera et al., 2019). An innovative feasible alternative to enhance the economic sustainability of the degradation and

valorization of CH_4 combined to the concomitant replacement of common plastics is the co-production of polyhydroxyalkanoates (PHAs) combined with the treatment of methane emissions (Cal et al., 2016; Pieja et al., 2017; Strong et al., 2016). Indeed, several studies have demonstrated that methanotrophs are a potential source of bioplastics, achieving PHA contents ranging from 20 to 60 % (wt) using methane as feedstock (Cantera et al., 2019; Pieja et al., 2017). Methanotrophs are divided into two different groups according to the pathway for carbon assimilation a) type I (γ-proteobacteria) use the ribulose monophosphate (RuMP) pathway or b) type II (α- proteobacteria) use of the serine pathway. Most of the studies reporting PHAs production using methanotrophic bacteria have been conducted with α-proteobacterial methanotrophs, since PHAs synthesis supposed to be linked with the serine cycle. Thus, acetyl-CoA molecules produced in the serine cycle are transformed into PHAs via reactions catalyzed by the enzymes β-ketothiolase (phaA),acetoacetyl-CoA reductase (phaB), and PHAs synthase (phaC) (Cantera et al., 2019). Overall, type II methanotrophic bacteria such as *Methylocystis, Methylosinus and Methylocella* have been considered the main methanotrophic PHA producing genera under nutrient-limited conditions (i.e N-, P- or Mg-limitation) (García-Pérez et al., 2018; Helm et al., 2008; Myung et al., 2017; Rostkowski et al., 2013; Sundstrom and Criddle, 2015; Wendlandt et al., 2001; Zhang et al., 2017).

However, the cost-effective implementation of current biotechnologies for the biotransformation of CH_4 into bioplastics is still limited by the limited understanding of those factors that determine the selective enrichment of high-performance PHA producing methanotrophs. In this sense, different operational parameters such as pH, CH_4/O_2 ratio, or the concentration of sodium, copper or citrate have been reported as key strategies for the

enrichment of type II methanotrophs with the ability to synthetize PHA (Pieja et al., 2011; Scheutz et al., 2009; Semrau et al., 2013). However, there is still a need for studies assessing the effect of environmental factors during culture enrichment of PHA producing methanotrophs. The use of an optimal temperature and the inoculum source have been reported as key strategies for the enrichment of microorganisms with the ability to synthetize PHAs using waste water and sugars as carbon sources (De Grazia et al., 2017). Thus, those inocula that favor the enrichment of type II methanotrophs, as well as the set temperature during enrichment could enhance this innovative biotechnology based on the transformation of methane into bioplastics. In this sense, *Sphagnum* mosses, which occur mainly in peat bogs, conifer forests and moist tundra areas, are described as the bryophytes with the highest richness of type II methanotrophs (Kip et al., 2011; Stępniewska and Kuźniar, 2014). Indeed, the extremophile environments that *Sphagnum* mosses inhabit, combined with the inherent presence of type II methanotrophs in their outer cortex, suggests their potential as a novel source of effective PHA-accumulating microorganisms (Kadouri et al., 2003; Ruiz et al., 2001; Zhao et al., 2007). Similarly, the stress caused on the metabolism of type II methanotrophs by an exposure to high temperatures might mediate an increase in PHA synthesis.

The study presented here aimed at systematically elucidating the influence of temperature (25ºC, 30ºC, 37ºC and 45ºC) during culture enrichment on the PHA synthetizing capacity, kinetics and stoichiometric characteristics, and population structure of microbial communities enriched using *Sphagnum* peat moss and a mixture of *Sphagnum* and activated sludge as inoculum.

2. Materials and Methods

2.1. Mineral salt medium and inoculum

The MSM (a modified NMS medium Whittenbury et al., (1970)) was composed of (g L^{-1}): 2.25 $NaNO_3$, 0.1 $MgSO_4 \cdot 7H_2O$, 0.02 $CaCl_2 \cdot 2H_2O$, 0.68 KH_2PO_4, 6.14 $Na_2HPO_4 \cdot 12H_2O$, 1.3×10^{-3} $FeSO_4 \cdot 7H_2O$, 3.5×10^{-3} $MnCl_2 \cdot 4H_2O$, 1.5×10^{-3} $ZnSO_4 \cdot 7H_2O$, 0.04×10^{-3} $Na_2MoO_4 \cdot 2H_2O$, 0.04×10^{-3} $CuSO_4 \cdot 5H_2O$, 0.32×10^{-3} $CoCl_2$, and 0.2×10^{-3} H_3BO_3. Moreover, vitamins (biotin, nicotinamid, p.aminobenzoic acid and panthotenic acid) were added to stimulate those methanotrophs that have problems to grow. All chemicals needed for MSM preparation were purchased from PANREAC (Barcelona, Spain), while CH_4 (≥ 99.5%) and O_2 (≥ 99.0%) were obtained from Abelló Linde S.A. (Barcelona, Spain). Poly [(R)-3-hydroxybutyric acid-co-(R)-3-hydroxyvaleric acid] (molar ratio 88/12, ≥ 99.99%) was purchased from Sigma-Aldrich® (St. Louis, MO, USA).

Fresh activated sludge from a denitrification-nitrification wastewater treatment plant (Valladolid, Spain) (López et al., 2018b) and *Sphagnum* peat moss from *Plantas Carnívoras*, (Madrid, Spain) were used as inocula for the enrichment of microorganisms able to degrade CH_4 and accumulate PHA. The *Sphagnum* peat moss was diluted 10 times in MSM prior inoculation.

2.2. Culture enrichment at different temperatures

A preliminary enrichment was performed batch-wise for 19 days at 25ºC in 1250 mL gas-tight bottles initially containing 190 mL of MSM and 20 ml of diluted *Sphagnum* inoculum (from now on referred as S) or 190 mL of MSM and 20 ml of diluted *Sphagnum* + activated sludge (50%/50%) (from now on referred as M). The initial mass of inoculum in each pre-enrichment was 50g/L. The bottles were closed with butyl septa and plastic caps, and the headspace was flushed with pure O_2 for 15 min in order to eliminate the previous air atmosphere. CH_4 was then supplied at an initial headspace concentration of 195 ± 7 g m^{-3}

and the bottles were incubated in an orbital shaker MaxQ 4000 fromThermo Scientific, at 200 rpm. Aliquots of 10 ml of the final enrichments S and M at 25ºC were transferred to 1250 mL gas-tight bottles prepared in duplicate as above described. Five enrichment cycles at 25, 30, 37 and 45ºC (each cycle involving a total CH_4 consumption and a complete headspace renewal) were carried out under orbital shaking at 200 rpm in duplicate. The temperature range of these experiments was chosen according to the optimum growth temperature of methanotrophic bacteria that are in general mesophile (Hanson and Hanson, 1996). No replacement of the MSM was conducted along the cycles, which entailed that the fifth cycle occurred under nitrogen limiting conditions. This procedure was designed to carry out an enrichment process under high biomass concentrations and low CH_4 aqueous concentrations (30 times lower than in the headspace according to the Henry´s law constant for CH_4 of 30 at 25ºC (Sander, 2014)) in order to favor the growth of methanotrophs with low apparent K_m values. In addition, a second enrichment in fresh MSM inoculated with 20 ml of the final cultivation broth resulting from the 5^{th}-cycle enrichment was conducted in duplicate at the different temperatures tested. The concentrations of CH_4, O_2 and CO_2 in the headspace and of TSS in the cultivation broth were periodically monitored in all enrichment experiments.

2.3. Influence of the temperature on CH_4 biodegradation kinetics

The kinetics study was performed in duplicate, batch-wise in gas-tight bottles (1250 mL) initially containing 200 mL of MSM and 7.3 mg of fresh biomass (obtained in the late exponential growth phase from a further culture inoculated with the final culture resulting from the second enrichments conducted in section 2.2). The serum bottles were hermetically closed, and the headspace was saturated with oxygen by flushing pure O_2 for

15 min. CH_4 was added to the headspace with gas tight syringes up to a final concentration of 194 ± 7 g m^{-3}. The serum bottles were then incubated in an orbital shaker at 25ºC, 30ºC or 37ºC and 200 rpm until methane degradation. CH_4, O_2 and CO_2 concentrations were periodically determined using a GC-TCD. In the case of the cell biomass it was measured as TSS drawing 3 ml from the cultivation broth. The specific growth rate (μ) was calculated from the slope of the logarithm of the biomass concentration *vs.* time. The specific CH_4 biodegradation rates were estimated from the ratio of the specific growth rates and the observed biomass yields in each experiment. The concentration of total nitrogen (TN) in the cultivation broth was also recorded. Neither significant CH_4 biodegradation nor biomass growth was recorded at 45ºC.

2.4. Influence of the temperature on PHAs accumulation under N-limiting conditions

The study was performed in duplicate batch-wise in 1250 mL gas-tight bottles initially containing 200 mL of N-free MSM and fresh biomass from the kinetics test (previously centrifuged and resuspended in N-free MSM). The bottles were closed with butyl septa and plastic caps, flushed with O_2 for 15 min and CH_4 was then supplied at an initial headspace concentration of 177 ± 7 g m^{-3}. The bottles were incubated in an orbital shaker) at 25ºC, 30ºC or 37ºC and 200 rpm until complete CH_4 depletion. In addition, fresh biomass enriched at 25 ºC was cultivated under nitrogen starvation conditions at 25, 30 and 37ºC, respectively, to elucidate the role of temperature during the accumulation phase. In this test series, the headspace concentrations of CH_4, O_2 and CO_2, biomass concentration (measured as TSS) in the cultivation broth and the PHB content of the biomass were periodically monitored.

174 *2.5. Analytical procedures*

175 CH_4, O_2 and CO_2 gas concentrations were determined using a Bruker 430 GC-TCD (Palo
176 Alto, USA) endowed with a CP-Molsieve 5A column (15 m × 0.53 µm × 15 µm) and a CP-
177 PoraBOND Q column (25 m × 0.53 µm × 10 µm). The oven, injector and detector
178 temperatures were 45 °C, 150 °C and 200 °C, respectively. Helium was the gas carrier at a
179 flow rate of 13.7 mL min^{-1}. Cell growth was measured as Total Suspended Solids (TSS)
180 according to Standards Methods (American Public Health Association (APHA) et al.,
181 2005). TN concentration was determined following sample filtration (0.45 µm) in a TOC-
182 VCSH attached to a TNM-1 module (Shimadzu, Japan). The bacterial PHB content was
183 measured using a GC–MS (GC System 7820A MSD 5977E, Agilent Technologies, Santa
184 Clara, USA) equipped with a DB-wax column (30 m × 250 µm × 0.25 µm) according to
185 (Frutos et al., 2017).

186

187 *2.6-DNA extraction, Illumina library preparation and 16S rRNA gene sequencing*

188 Amplicon sequencing was developed targeting the 16S V3 and V4 regions (464bp,
189 *Escherichia coli* based coordinates) with the bacterial primers S-D-Bact-0341-b-S-17 and
190 S-D-Bact-0785-a- A-21, forward and reverse, respectively according to (Klindworth et al.,
191 2013). Universal primers were used instead of specific primers for methanotrophic bacteria
192 with the aim of having a broad view of the bacterial population that could be implied in
193 PHA production. Illumina adapter overhang nucleotide sequences were added to the gene-
194 specific sequences according to (López et al., 2018b). Library construction was carried out
195 using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA). Libraries
196 were then normalized and pooled prior to sequencing. Non-indexed PhiX library (Illumina,

San Diego, CA) was used as performance control. Samples containing indexed amplicons were loaded onto the MiSeq reagent cartridge for automated cluster generation paired-end sequencing with a 2x300pb paired-end run (MiSeq Reagent kit v3 (MS-102-3001)) according to manufacturer's instructions (Illumina). The amplicon sequencing analysis was carried by the Foundation for the Promotion of Health and Biomedical Research of Valencia Region (FISABIO, Spain).

2.7-16S rDNA-based taxonomic analysis

Only reads with quality value scores ≥20 in more than 99% of the sequence after demultiplexing and without ambiguous base calls were further analyzed. Quality analyses, paired-end reads junction and chimera search were carried out using the prinseq-lite program (Schmieder and Edwards, 2011), the FLASH program (Magoc and Salzberg, 2011) and the USEARCH program (Edgar, 2010). Taxonomic assignments were then conducted using the RDP- Classifier from the Ribosomal Database Project (Cole et al., 2009; Wang et al., 2007). Simpson and Shannon indices were determined using the Vegan library version 2.3e1 (Oksanen et al., 2015). The Krona tool was used to represent relative abundances and confidences within the complex hierarchies of metagenomics classifications (Ondov et al., 2011). Nucleotide sequence dataset obtained in this study has been submitted to NCBI Sequence Read Archive with the bioproject accession PRJNA521405 (https://trace.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA521405&go=go).

2.8-Statistical Analyses

All analyses were performed using biomass obtained from two biological replicas for each condition. The specific growth rates, biomass yields, specific CH_4 biodegradation rates, PHAs contents and PHAs produced per unit of CH_4 consumed, were compared pairwise using a single cued t-test assuming equal variance. The differences were considered statistically significant for p-values below 0.05. The symbols "*", "**" and "***" were used to indicate values below 0.05, 0.01 and 0.001, respectively, in the figures. In the particular cases where two conditions were not significantly different from each other, their values were grouped and a t-test was performed compared to a third condition. Vegan (Oksanen et al., 2015) and stats packages in R (Sasaki et al., 2005) were used for the statistical analyses of the metagenomics data.

3. Theoretical Framework

3.1. Stoichiometry of PHA production

PHA production using methane as the sole carbon and energy source requires a biomass formation step under nitrogen excess conditions followed by a PHA accumulation step under nitrogen limiting conditions. This two-stage process can be implemented in two separate units (Figure S1). The CH_4 and nitrogen required to produce a unit of PHA (disregarding CH_4 maintenance consumption, which is small compared to its consumption associated biomass and PHA formation) will depend on the biomass yield on CH_4 ($Y_{X/CH4}$), the potential PHA content accumulated per unit of biomass ($F_{PHA}=m_{PHA}/X$; X being the initial biomass without PHA accumulation), the PHA yield on CH_4 ($Y_{PHA/CH4}$) and the fraction of nitrogen contained in the biomass before PHA accumulation (F_N). Thus, the production of one unit of PHA requires the previous formation of an amount of biomass X

equal to $1/F_{PHA}$, which itself requires a CH_4 supply equal to $X/Y_{X/CH4}$ in the first unit (under nitrogen excess). On the other hand, the amount of CH_4 to be supplied to the second unit (under nitrogen limiting conditions) could be estimated as $1/Y_{PHA/CH4}$. Therefore, the total methane supplies necessary to obtain a mass unit of PHA (m_{CH4}) can be calculated as $1/(F_{PHA}Y_{X/CH4}) + 1/Y_{PHA/CH4}$. Likewise, the nitrogen required per unit of PHA m_N is equal to $F_N/(F_{PHA})$.

3.2. Biodegradation kinetics and gas-liquid CH_4 transfer

Both the biomass formation and PHA accumulation units require the transfer of CH_4 (and oxygen) from the gas emission to the methanotrophic cultivation broth. Under steady state operation, the rate of CH_4 and O_2 mass transfer per unit of reactor volume will be equal to the volumetric rate of microbial consumption of these substrates. The rate of CH_4 mass transport per volume of reactor (R_{CH4}) can be described by the following equation (Bordel et al., 2008) (Eq. [1]):

$$R_{CH_4} = \frac{Q_g}{V}\beta_{CH_4}\left(C_{gin} - H_{CH_4}C_L\right) \quad [1]$$

Where C_{gin} and H_{CH4} stand for the inlet concentration and Henry's law constant of CH_4, V the bioreactor volume, Q_g the gas flow rate and C_L the CH_4 aqueous concentration in the cultivation broth. The parameter β_{CH4} ranges between 0 and 1, and depends mainly on the gas-liquid interface area in the reactor as well as the transport properties of the system (Bordel et al., 2008) (Eq. [2]).

$$\beta_{CH_4} = 1 - exp\left(\frac{-k_L a V}{H_{CH_4} Q_g}\right) \quad [2]$$

Where $k_L a$ represents the volumetric mass transfer coefficient of CH_4 in the bioreactor. The value of the parameter β_{CH4} is equal to the maximum fraction of CH_4 transferrable from the gas emission to the cultivation broth within the reactor. This parameter is independent from

the biological parameters of the microbial community present in the cultivation broth. Therefore, it is desirable to operate with β_{CH4} values close to 1. However, low β_{CH4} values are typically recorded for gas substrates with a low aqueous solubility such as CH_4 (e.g the dimensionless Henry constant H_{CH4} is equal to 33.5 at 30 ^{o}C (Duan et al., 1992)), which would ultimately limit the performance of PHA production based on CH_4. In this context, several high mass transfer performance bioreactors such as two-phase partitioning, Taylor flow or internal gas recycling bioreactors have been engineered to overcome these limitations (Bordel et al., 2010; García-Pérez et al., 2018; Hernández et al., 2011). However, the kinetic parameters of the methanotrophic community influence the CH_4 mass transport rate since microbial kinetics ultimately determine the steady state liquid concentration (C_L). The specific biomass growth rate of methanotrophs (μ) can be accurately described using the Monod´s equation (Eq. [3]):

$$\mu = \mu_{max} \frac{C_L}{C_L + K} \quad [3]$$

Where μ_{max} stands for the maximum specific growth rate and K the Monod half-saturation constant. The specific biomass growth rate in the biomass formation unit under steady state is equal to the dilution rate D (= volumetric liquid flow rate supplied to the reactor divided by the reactor volume). Hence, the CH_4 aqueous concentration, C_L, can be determined as a function of the dilution rate (Eq. [4]).

$$C_L = \frac{DK}{\mu^{max} - D} \quad [4]$$

The Monod constant K is equal to the concentration at which the cell growth rate is equal to half its maximum value (μ_{max}). The value of K in methanotrophic cultures typically ranges between 2 and 12 μM. On the other hand, the maximum specific growth rate sets a threshold to the dilution rate of the bioreactor. Thus, the dilution rate should be at least an

288 order of magnitude lower than this critical dilution rate in order to cope with increases in
289 the necessary liquid flow to be processed and to avoid biomass wash-out. Based on
290 equation [1], the fraction (F_{CH4}) of CH_4 in the gas emission transferred to the cultivation
291 broth can be estimated as follows (Eq. [5]):

292 $$F_{CH4} = \beta_{CH4} \frac{C_{gin} - H_{CH4} C_L}{C_{gin}} \quad [5]$$

293 In this context, while the parameter β_{CH4} depends only on the gas-liquid mass transfer
294 capacity of the bioreactor, C_L is governed by the liquid dilution rate and the kinetics
295 parameters of the microbial community. Therefore, the fraction F_{CH4} can be regarded as the
296 product of a transport limited term and a biologically limited fraction. This biologically
297 limited term is close to 1 for the typical kinetics and operating parameters prevailing in CH_4
298 abatement bioreactors, which entails that the impact of the maximum specific biomass
299 growth rate on the overall process performance is small.
300
301 **4. Results**
302 *4.1. Influence of the enrichment temperature on the specific growth rates*
303 A decrease in the specific growth rate of the microbial communities was observed when
304 increasing the selected temperature in the enrichments derived from *Sphagnum* alone at a
305 significant level of $p \leq 0.05$ (Figure 1). Thus, average specific growth rates of 0.05±0.001
306 h^{-1}, 0.04±0.004 h^{-1} and 0.02±0.004 h^{-1} were observed at 25, 30 and 37ºC, respectively, in S
307 enrichments. However, the specific growth rates of the microbial communities enriched
308 from the mixed inoculum (M) increased from 0.02±0.002 h^{-1} at 25^{o}C to 0.033±0.0009 h^{-1} at
309 37^{o}C. Interestingly, the specific growth rates of the two M biological replicates enriched at

310 30°C differed strongly (0.022 and 0.042 h^{-1}), resulting in no statistical differences with the
311 M enrichments at 25 and 37ºC.

312 *< Figure 1>*

313 *4.2. Influence of the enrichment temperature on the specific CH_4 degradation rate*

314 No significant difference was observed between the specific CH_4 biodegradation rates of
315 the biomass enriched from *Sphagnum* at 25 and 30 °C (65.3 ±5.1, 79.5 ±7.9 mg-CH_4 h^{-1} g-
316 $biomass^{-1}$, respectively; error intervals are standard errors with 2 samples) (Figure 2).
317 However, a significantly lower specific CH_4 biodegradation rate was observed at 37°C
318 (49.7 ±3.7 mg-CH_4 h^{-1} g-$biomass^{-1}$), which correlated with the lower μ recorded at this
319 temperature. On the other hand, the biomass enriched from the mixed inoculum (M)
320 exhibited lower specific CH_4 biodegradation rates at 25 and 30°C (48.2 ±2.5 and 51.7 ±1.9
321 mg-CH_4 h^{-1} g-$biomass^{-1}$, respectively) than at 37°C (78.9 ±5.6 mg CH_4 h^{-1} g $biomass^{-1}$). The
322 differences obtained between the specific CH_4 biodegradation rates of S and M confirmed
323 that the experiments were conducted under non-mass transfer limiting conditions.

324 *<Figure 2>*

325

326 *4.3. Influence of the enrichment temperature on PHAs content*

327 The content of biopolymers accumulated by the microorganisms of both inocula increased
328 with temperature. Polyhydroxybutyrate was the dominant PHA in all the samples analysed.
329 The biomass enriched at 30 and 37°C was able to accumulate over 30% of PHA inside the
330 cells (35.1 ± 0.4% and 33.1± 1.3% in S at 30ºC and 37ºC, respectively, and 28.4 ± 1.1%
331 and 34.1± 1.3% in M at 30ºC and 37ºC, respectively) under nitrogen limiting conditions,
332 compared to the PHA contents of 15.7 ± 2.4% and 21.2± 2.0% in S and M biomass,

respectively, at 25ºC (Figure 3). The mass of PHA per unit of CH_4 consumed was also obtained from experiments conducted under N limiting conditions. Interestingly, no significant difference was observed among the 6 microbial communities enriched, with average values of 0.22 ± 0.05 g-PHA $(g\text{-}CH_4)^{-1}$. In this context, this value has been previously reported to be 0.57 g-PHA $(g\text{-}CH_4)^{-1}$ for pure cultures of *Methylocystis hirsuta* , which is a high-performance PHA accumulating methanotrophic strain (López et al., 2018a). Similarly, Chidambarampadmavathy et al. (2017) , using a consortium enriched from landfill soil and dominated by *Methylosarcina sp*., obtained maximal PHB contents of 25 mg g biomass-1 at 40% of CH_4 in air. Zhang et al., 2018 reported accumulations of ~45% of PHB in a mixed culture enriched from sewage sludge under N limitation and with Cu, whereas the absence of Cu diminished PHB synthesis (12%–18%).”

<*Figure 3*>

4.4 Influence of the enrichment temperature on biomass yield

The biomass enriched from *Sphagnum* at 25ºC showed a yield on CH_4 almost twice as high as the biomass enriched from this inoculum source at the two other temperatures (Figure 4). This higher yield was also correlated to the higher specific growth rate observed at this temperature. As discussed later, this higher yield might be somehow related to the higher population diversity observed in this sample. The biomass derived from the mixed inoculum at 25ºC did not show yields higher than that enriched at 30 and 37ºC. In contrast, the higher yield on CH_4 was recorded in the biomass enriched at 30ºC.

<*Figure 4*>

356 *4.5. Selection of optimal enrichment conditions for PHA accumulation*

357 From an industrial perspective, this study aimed at elucidating the optimal enrichment
358 temperature and inoculum source that potentially result in the most effective usage of raw
359 materials (in this case CH_4 and N). Table 1 depicts the CH_4 and nitrogen required to
360 produce a unit of PHA as a function of the enrichment temperature and inoculum source.
361 The most cost-effective biomass for optimal PHA production would be the enrichment
362 from *Sphagnum* at 30^{o}C. This biomass required the lowest CH_4 input (10.5±1.4 g CH_4 g
363 PHA^{-1}; equal to that required by the biomass obtained at 30^{o}C from the mixed inoculum)
364 and also the lowest nitrogen input (0.2 g N g PHA^{-1}).

365 <*Table 1*>

366

367 *4.6. Influence of the temperature during the accumulation phase on PHA content*

368 The results herein obtained showed that, independently of the inoculum source, the biomass
369 enriched at 30 and 37^{o}C led to the highest PHA yields. In the previous experiments in
370 literature, biomass enrichment and PHA synthesis (under nitrogen limitation) were carried
371 out at a constant temperature (García-Pérez et al., 2018; López et al., 2018a, 2018b; Myung
372 et al., 2017; Zhang et al., 2017). In order to elucidate whether these higher PHA yields were
373 the result of the temperature during the enrichment or accumulation phase, the biomass
374 enriched at 25^{o}C was supplied with CH_4 under nitrogen starvation conditions at 25, 30 and
375 37^{o}C. No increase in the PHA content of the biomass enriched at 25ºC was observed at the
376 two highest temperatures. Indeed, a slight decrease (no statistically significant) was found
377 at 30 and 37ºC (Figure 5).

378 <Figure 5>

379

4.7. Influence of the enrichment temperature on the structure of the microbial communities

The enrichment process (pre-enrichment + 5 growth stages till complete N depletion + 2 growth stages + 1 PHA accumulation stage) resulted in the dominance of the genus *Methylocystis* (with percentages close to 30%, Figure S2) in the enriched communities regardless of the inoculum source and temperatures. Members of this genus, which was almost absent in the initial inocula, are capable of synthetizing PHA under nitrogen deprivation conditions (López et al., 2018a). The biomass enriched from *Sphagnum* contained a low number of other methanotrophs and methylotrophs (~1% of *Methylobacterium* at 30 and 37^{o}C, and 2% of *Methylophylus* at 25^{o}C). On the other hand, the biomass enriched from the mixed *Sphagnum* and activated sludge inoculum contained significant amounts of methylotrophs such as *Methylophilus* (17% of the community enriched at 25^{o}C), *Methylobacterium* (5% of the community enriched at 30^{o}C) and methanotrophs such as *Methyloparacocus* (9% of the community enriched at 30^{o}C). These methanotrophs and methylotrophs different from the genus *Methylocystis* were less abundant in the community enriched at 37^{o}C (1% of *Methylobacterium*). The results here obtained revealed that temperature was a strong selective pressure for the enrichment of PHA accumulating bacteria regardless of the inoculum.

None of the characteristics of the microbial communities enriched (specific growth rate, PHA accumulation etc.) seemed to be explained by changes in the overall microbial composition. For example, the enrichments conducted at 30 and 37ºC, which resulted in higher PHA accumulations, did not contain a higher percentage of *Methylocystis* than those conducted at 25ºC.

5. Discussion

403 *5.1. Effect of the enrichment conditions on the microbial community profile*

404 This study aimed at assessing both the feasibility of enriching PHA accumulating
405 methanotrophic consortia from *Sphagnum* mosses-based inocula (based on their high
406 inherent abundance in type II methanotrophs) and the influence of the temperature on the
407 characteristics of the communities enriched. The prolonged exposure of the inocula to
408 methane as the only carbon and energy source (following the described enrichment
409 protocol) allowed to enrich microbial consortia where the genus *Methylocystis* was the most
410 abundant (which represented approx. 30% of the total microbial population) regardless of
411 the enrichment temperature. *Methylocystis* are known to accumulate PHA, however no
412 selective pressure aiming to favour PHA accumulating organisms (such as alternate cycles
413 of nitrogen starvation) was specifically imposed during the enrichment process. The
414 specific growth rates of the consortia enriched were up to two folds higher than those
415 previously reported for pure cultures of *Methylocystis hirsuta* (López et al., 2018a) (0.021
416 h^{-1}), likely due to the symbiotic effects with other microbial populations. In this context, the
417 purer a culture is, the higher are the restrictions to grow under environmental stress factors,
418 while a higher population richness and diversity promotes higher resilience and therefore,
419 better bio-product recovery (Cabrol et al., 2012). Moreover, adaptive laboratory evolution
420 could be also carried out to gain insights into the adaptive changes that experience
421 microbial populations during long-term selection: better PHA productivity and higher
422 methane degradation rates (Dragosits and Mattanovich, 2013).

423

424 *5.2. Specific growth rate and microbial CH_4 bioconversion*

425 In addition, the theoretical framework developed in section 3 reveals that a microbial
426 community with a maximum specific growth rate of 0.02 h^{-1} (the minimum recorded in this

427 study) treating a gas emissions with typical CH_4 inlet concentration of 6% (equivalent to an
428 equilibrium concentration of 0.0024 M in the aqueous phase) will eventually support a CH_4
429 removal of 99.2% in the bioreactor (under no mass transfer limitations), while a biomass
430 with a maximum specific growth rate of 0.05 h^{-1} (the maximum recorded in this study) will
431 support a potential CH_4 removal of 99.7%. These estimations clearly show that the
432 maximum specific growth rate of the microbial community enriched does not represent a
433 key parameter in suspended-growth CH_4 bioconversion processes operated under
434 continuous mode. The scenario would be different if the process was performed batch-wise,
435 where higher maximum specific growth rates would result in shorter production times.
436 The biomass yields on methane here recorded were of the same order as those of pure
437 cultures of *Methylocystis*, with the exception of the consortium enriched from the inoculum
438 S at 25ºC, which showed a biomass yield almost twice as large as that of a pure
439 *Methylocystis hirsuta* cultures. It can be hypothesized that this unusually high yield
440 (YX/CH_4 =0.8) was supported by the high microbial diversity of this particular enrichment
441 (H = 2.84). A weak correlation between the biomass yield and the microbial diversity was
442 also observed (R=0.764 and a p-value=0.109), which has been previously described in
443 literature (Louis et al., 2016).
444
445 5.3 Effect of temperature on the enrichment conditions and on PHA accumulation
446 Since temperature has been determined as an important factor that modifies PHA
447 accumulation in other heterotrophic bacteria (Myshkina et al., 2008), this study aimed to
448 assess the optimal temperature conditions to increase the production of PHA using type II
449 methanotrophs. Culture enrichment at 30 and 37ºC resulted in consortia able to accumulate

450 significantly higher amounts of PHA under nitrogen starvation (30% of the total microbial
451 biomass) than those enriched at 25ºC. These values were low in comparison to the PHA
452 contents of 40-47% (wt) reported by Zhang et al. 2018. This result was probably due to the
453 high copper concentration used for enrichment by Zhang et al. 2018 which increased PHA
454 accumulation by a factor of 2.5. Concentrations of copper characteristic of urban waste
455 water treatment plants were used in this study to avoid the effect of additional variables on
456 the results obtained not related to temperature (Zhang et., 2018). Nevertheless, the fact that
457 only 30% of the DNA of the enriched consortia corresponded to bacteria of the genus
458 *Methylocystis* (the only described PHA accumulating organism identified in these
459 consortia) suggested that the strains of this genus in these specific enrichments could
460 eventually contain up to 70% of PHA. In this regard, under nutrient-limited conditions
461 *Methylocystis*, *Methylosinus* and *Methylocella*, which are considered the main
462 methanotrophic PHA producers to date, are able to achieve PHA accumulations ranging
463 from 20 to 50% (wt) (García-Pérez et al., 2018; Pieja et al., 2012; Zhang et al., 2017)
464 values that are lower than the ones suggest by previous research.
465 The fact that the results obtained for the PHA content of the microbial communities
466 enriched at different temperatures were just due to the temperature prevailing during the
467 accumulation phase was discarded by performing PHA accumulation experiments at 25, 30
468 and 37ºC with biomass enriched from *Sphagnum* at 25ºC. No enhancement in PHA
469 accumulation was observed at 30 and 37ºC, which confirmed that culture enrichment at
470 higher temperatures plays a key role on the selection of more efficient PHA accumulators.
471 However, the mechanisms underlying the superior accumulation of PHA in communities
472 enriched at higher temperatures remains unknown. In this context, it has been speculated
473 that temperature acts as an environmental stress analogous to nitrogen depletion and

474 triggers PHA accumulation. However, only trace levels of PHA were observed at 30 or
475 37ºC in cultures grown under nitrogen sufficient conditions and there was not a direct
476 influence of temperature on PHA accumulation under nitrogen limiting conditions during
477 the accumulation phase. At this point it can be speculated that some molecular mechanisms
478 involved in heat shock resistance might also enable methanotrophs to accumulate higher
479 PHA contents. Finally, it should be highlighted that lower temperatures increase CH_4 mass
480 transfer from the gas to the microbial community as a result of a decrease in the Henry's
481 law constant, which directly impact on the gas-liquid concentration gradient and could
482 influence the specific growth rates and PHA content of the biomass. However, the
483 theoretically enhanced CH_4 mass transfer at lower temperatures did not increase the PHA
484 content of the microbial communities enriched.
485
486 **6. Conclusions**
487 This study confirmed the key role that the enrichment temperature plays on microbial PHA
488 production from CH_4, culture enrichment at high temperatures (30 and 37ºC) resulted in a
489 superior PHA accumulation compared to the biomass enriched at 25ºC. This finding
490 suggests that temperature has an important in PHA accumulation and could enhance the
491 implementation of PHA cell factories.
492
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Table 1. Methane and nitrogen requirements and biomass production per gram of PHA produced.

	Sphagnum			**Sphagnum + Activated sludge**		
	25 °C	**30 °C**	**37 °C**	**25 °C**	**30 °C**	**37 °C**
Biomass per gram of PHA (g)	6.3±0.6	2.8±0.04	3±0.05	4.7±0.2	3.5±0.2	2.9±0.1
CH_4 growth phase (g)	4.5±1	4.5±1	4.5±1	4.5±1	4.5±1	4.5±1
CH_4 accumulation phase (g)	7.9±2.4	6±0.4	7±1.3	10.5±2	5.8±1	6.8±1.4
Total CH_4 per gram of PHA (g)	12.5±3.4	10.5±1.4	11.6±2.3	15±3	10.3±2	11.4±2.4
Nitrogen per gram of PHA (g)	0.5±0.1	0.2±0.0	0.2±0.0	0.3±0.0	0.3±0.0	0.2±0.0

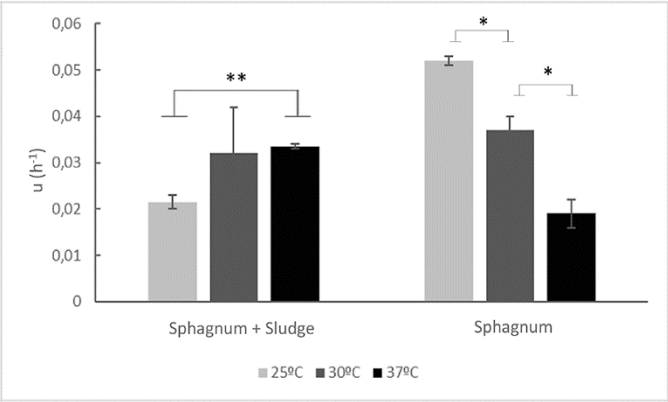

Figure 1. Influence of the enrichment temperature on the specific growth rates for the communities enriched from *Sphagnum* and *Sphagnum* + activated sludge. For the mixed *Sphagnum* and activated sludge inoculum, error bars show standard errors (n=2), and the specific growth rate of the biomass enriched at 37 ^{o}C was significantly higher than the

722 growth rate at 25 ^{o}C (p-value 0.008, n=2). For *Sphagnum* inoculum, the opposite trend was
723 observed, with decreasing specific growth rates at higher temperatures (p-values of 0.02
724 between 25 and 30 ^{o}C and 0.03 between 30 and 37 ^{o}C, n=2).
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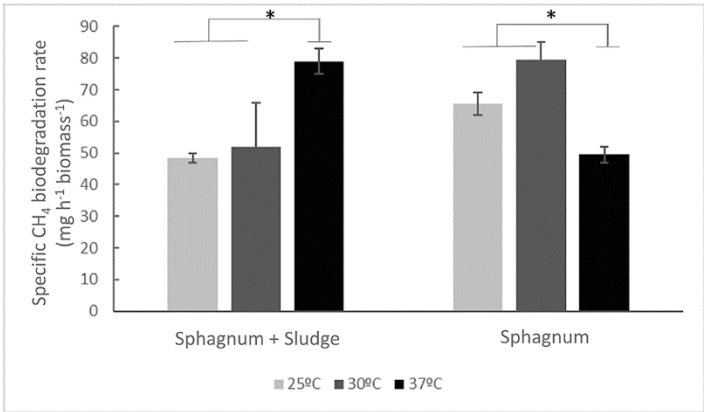

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730 **Figure 2.** Influence of the enrichment temperature on the specific CH_4 biodegradation rate
731 for the communities enriched from *Sphagnum* and *Sphagnum* + activated sludge. Error bars
732 are standard errors (n=2). In both cases, no significant difference is observed between the
733 enrichment at 25 and 30 ^{o}C. The biomass enriched at 37 ^{o}C showed a significant increase
734 (p-value 0.017; t-test for samples of different sizes n=2, m=4) for the mixture of *Sphagnum*
735 and activated sludge and a significant decrease (p-value 0.018; t-test for samples of
736 different sizes n=2, m=4) for *Sphagnum* alone.
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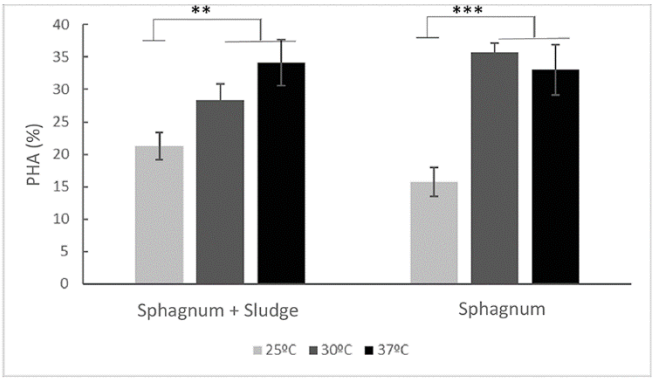

Figure 3. Influence of the enrichment temperature on the PHA content of the biomass enriched from *Sphagnum* and *Sphagnum* + activated sludge. Error bars represent standard errors (n=2). The same trend was observed for the mixture of *Sphagnum* and activated sludge and *Sphagnum* alone. Significantly higher PHA contents were observed at 30 and 37 ^{o}C compared to 25 ^{o}C (p-values of 0.00015 for *Sphagnum* and 0.005 for the mixture; t-test for samples of different sizes n=2, m=4). The differences between 30 and 37 ^{o}C were not statistically significant.

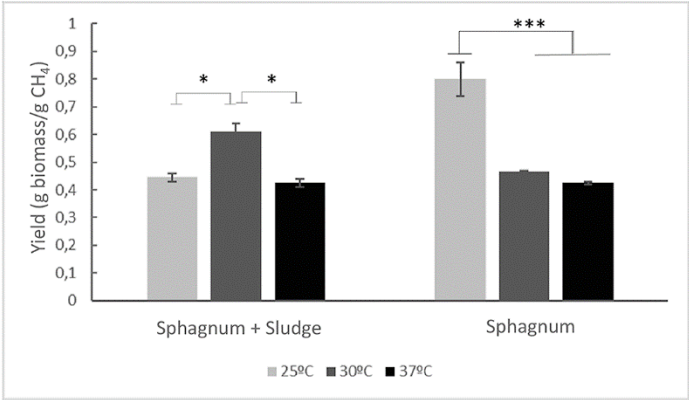

Figure 4. Influence of the enrichment temperature on the biomass yield on CH_4 from *Sphagnum* and *Sphagnum* + activated sludge. Error bars represent standard errors (n=2). For the mixture of *Sphagnum* and activated sludge, the biomass enriched at 30 ^{o}C showed a significantly higher yield (p-value 0.012; t-test for samples of different sizes n=2, m=4) compared to the biomass enriched at 25 and 37 ºC. For the *Sphagnum* alone a very significant increased yield was observed for the biomass enriched at 25 ^{o}C (p-value 0.00097; t-test for samples of different sizes n=2, m=4).

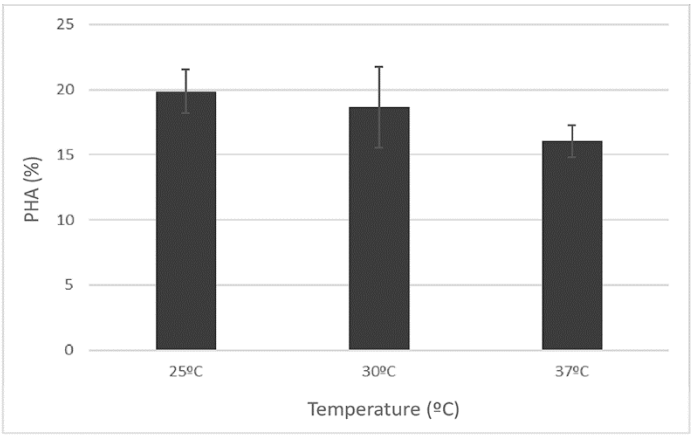

Figure 5. Influence of the temperature during the accumulation phase on the PHB content of the biomass enriched from *Sphagnum* at 25 ºC under nitrogen starvation at 25, 30 and 37 ºC, respectively. Error bars are standard errors (n=2).