Multi-production of high added market value metabolites from diluted methane emissions via methanotrophic extremophiles

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Abstract

This study is the first proof of concept of a methane biorefinery based on the multi-production of high profit margin substances (ectoine, hydroxyectoine, polyhydroxyalkanoates (PHAs) and exopolysaccharides (EPS)) using methane as the sole carbon and energy source. Two bubble column bioreactors inoculated with a pure strain (*M. alcaliphilum 20Z*) and a halophilic methanotrophic consortium (mainly composed of *Halomonas*, *Marinobacter*, *Methylophaga* and *Methylomicrobium*) were operated under different magnesium concentrations (0.2, 0.02 and 0.002 g L^{-1}) with the aim of validating and optimizing this innovative strategy for the valorization of dilute CH_4 emissions. High Mg^{2+} concentrations promoted the accumulation of ectoine (79.7-94.2 mg g $biomass^{-1}$), together with high hydroxyectoine yields (up to 13 mg g $biomass^{-1}$) and EPS (up to 2.6 g g $biomass^{-1}$). Unfortunately, PHA synthesis was negligible at 0.2 and 0.02 g Mg^{2+} L^{-1}, and supported low PHAs concentrations (14.3 mg L^{-1}) at the lowest Mg^{2+} concentrations. The elimination capacities of methane in both bioreactors

ranged from 15 to 20 g m^{-3} h^{-1} at 0.2 and 0.02 g Mg^{2+} L^{-1}, even if the most representative genera have not been previously identified as methanotrophs before. The promising results here obtained encourage further research on CH_4 biorefineries capable of creating value out of GHG mitigation.

Keywords: CH_4-biorefinery, ectoines, exopolysaccharides, *Halomonas*, methane abatement.

Introduction

Methane (CH_4) is nowadays the second most important greenhouse gas (GHG) as a result of its high global warming potential (85 times higher than that of CO_2 over a 20-y window) and emission rates. Despite CH_4 can be used as an energy vector for electricity and heat generation when its concentration in the gas emission is higher than 20 %, more than 60 % of anthropogenic CH_4 emissions worldwide contain concentrations lower than 4 %[1,2]. When applied to these diluted gas emissions (such as off-gases from landfills or coal mines), state-of-the-art physical/chemical technologies for CH_4 abatement are neither environmentally friendly nor cost-effective[3].

Nowadays, the lack of a suitable approach to prevent the adverse environmental effects of CH_4 has encouraged both political initiatives to control these GHG emissions and an intensive research on novel strategies for CH_4 abatement [4]. Of them, the bioconversion of CH_4 into high added value products using a bio-refinery approach has emerged as one of the most promising ones[5–7]. In this regard, CH_4-laden emissions can be used by methanotrophs as feedstock to synthesize bioproducts with a high profit margin, such as

biopolymers, exopolysaccharides or ectoines, turning CH_4 emissions abatement into a sustainable and profitable process.

Ectoine and its hydroxylated derivative (hydroxyectoine) are one of the most valuable bioproducts synthesised by microorganisms, retailing in the pharmaceutical industry at approximately US$1000 kg^{-1}. These compatible solutes, which are synthesized by bacteria to survive in salt-rich environments, are effective stabilizers for enzymes, DNA-protein complexes and nucleic acids[8]. Recent studies have demonstrated that *Methylomicrobium alcaliphilum 20Z*, an alkalophilic and halotolerant methanotroph, is able to produce ectoine at 37-70 mg L^{-1} under continuous CH_4 fermentation[9]. However, the ectoine productivities obtained in bioreactors by methanotrophs are still low in comparison with those reported for heterotrophic bacteria such as *Halomonas elongate* and *Halomonas salina*[8]. Moreover, the halophilic methanotrophs discovered to date are sensitive to shear stress, which entails the need for bioreactor operation at low agitation rates, thus hampering the mass transfer of CH_4 to the microbial community[10]. Finally, it should be stressed that hydroxyectoine accumulation by methanotrophs has never been reported to date[11].

Polyhydroxyalkanoates (PHAs) are intracellular biopolyesters produced under nutrient-limiting and carbon-excess conditions as carbon and energy storage resources by a wide range of microorganisms [12]. Their outstanding mechanical properties, along with their biodegradability and biocompatibility, turns PHAs into an attractive and potential alternative to oil-based plastics[5,13]. Under nutrient-limiting conditions (i.e N-, P- or Mg-limitation), methanotrophic bacteria are able to reach PHAs accumulations ranging from 20 up to 50 % (on a dry weight basis) in suspended growth bioreactors operated under batch[14,15] and continuous mode[16].

On the other hand, extracellular polysaccharides (EPS) constitute another potential high added value bioproduct resulting from CH_4 biorefineries. These biopolymers, in which biofilms are embedded, are composed of a wide variety of proteins, glycoproteins, glycolipids and polysaccharides[17]. EPS are typically excreted by bacteria under stress conditions as a protective barrier or water retainer. The interest in these novel bioproducts derive from their colloid and adhesive properties, and their effects on liquid rheology, gaining relevance in the food, pharmaceutical, textile and oil industries[18]. To date, some studies have demonstrated that methanotrophic bacteria are able to synthesize EPS in the range of 0.03-0.43 g $g^{-1}_{biomass}$[19].

In this context, the present study represents an assessment of the potential of the continuous bioconversion of CH_4 into multiple added-value products (i.e ectoine, hydroxyectoine, PHAs and EPS) as an innovative strategy for the valorization of diluted CH_4 emissions. For this purpose, a systematic comparison of the performance of two bubble column bioreactors inoculated with haloalkaliphilic methanotrophs, a pure strain *M. alcaliphilum* 20Z and an enriched haloalkaliphilic consortium, was carried out. The influence of magnesium (Mg^{2+}) concentration on the synthesis of the target bioproducts and on the structure of the bacterial communities was evaluated.

Materials and Methods

2.1. Chemicals and mineral salt medium

The mineral salt medium (MSM) used for the cultivation of the haloalkaliphilic methanotrophs in this study was a high pH (9.0) and high-salt content (6% NaCl) medium recommended for the enrichment of methane oxidizing bacteria from soda lakes.[20] Magnesium was supplemented to the MSM in the form of $MgSO_4$ at the 3

concentrations tested (0.2 (C1), 0.02 (C2), 0.002 (C3) g L^{-1}). The limitation of Mg^{2+} in C2 and C3 was carried out with the aim of increasing PHAs production according to Khanna et al. (2005)[21]. Nitrogen limitation was not tested due to the negative effect on ectoine and hydroxyectoine production. All chemicals and reagents were obtained from Panreac (Barcelona, Spain) with a purity higher than 99.0 %. CH_4 (purity of at least 99.5 %) was purchased from Abello-Linde S.A (Barcelona, Spain).

2.2. Microorganisms and inocula preparation

Reactor 1 (R1) was inoculated with a pure strain of *M. alcaliphilum 20Z* acquired from DSMZ (Leibniz-Institut). In brief, a 10× dilution of the *M. alcaliphilum 20Z* stock culture from DSMZ was grown at 25 ºC in 120 mL sterile glass bottles containing 40 mL of MSM at 0.2 g $MgSO_4$ L^{-1}. The bottles were closed with gas-tight butyl septa and aluminum caps, and 50 % (v/v) of the air headspace was replaced by CH_4. The inoculum, which was grown up to a biomass concentration of 0.1 ± 0.06 g, was transferred to two sterile gas-tight glass bottles (1.2 L) closed with butyl septa and plastic screw caps, and containing 180 mL of MSM at 0.2 g $MgSO_4$ L^{-1} (20 mL per bottle) prior reactor inoculation. CH_4 was injected to obtain a CH_4 headspace concentration of 55.0 ± 6.2 g CH_4 m^{-3}. The magnetic agitation rate was set at 600 rpm and the temperature used was 25 ºC.

Reactor 2 (R2) was inoculated with an enrichment of haloalkaliphilic bacteria able to grow using methane as the only external carbon and energy source. Fresh activated sludge from a denitrification-nitrification wastewater treatment plant with seawater intrusion (Cantabria, Spain) and fresh cow manure and soil from a dairy farm on the coastline of Cantabria (Spain) were used as inoculum for the enrichment. Culture enrichment was carried in two sterile gas-tight glass bottles (1.2 L) containing 190 mL of MSM at 0.2 g $MgSO_4$ L^{-1} inoculated with 10 mL of the cow manure-soil mixture and

10 mL of the activated raw sludge. The bottles were closed with butyl septa and plastic screw caps, and CH_4 was injected to obtain a CH_4 headspace concentration of 55.1 ± 2.7 g CH_4 m^{-3}. The enrichments were transferred 7 times to fresh medium bottles upon CH_4 depletion using 10 % inoculum aliquots. The magnetic agitation rate was set at 600 rpm and the temperature used was 25 ºC.

*2.*3 *Experimental set-up and operating conditions*

Two 2.0 L bubble column reactors (Afora S.A., Spain) were used for continuous CH_4 abatement combined with the co-production of ectoines, EPS and PHAs. The influence of three different Mg^{2+} concentrations (0.2 g L^{-1}, 0.02 g L^{-1} and 0.002 g L^{-1}) on production of the above mentioned bioproducts was assessed in both reactors during stages C1, C2 and C3, respectively. R1 was inoculated with *M. alcaliphilum 20Z* at an initial concentration of 520 mg L^{-1}, while R2 was inoculated with the haloalkaliphilic bacteria enrichment at 500 mg L^{-1}. A 0.066 L min^{-1} CH_4-laden air emission containing 25.9 ± 2.1 g CH_4 m^{-3} (≈ 4 %), corresponding to a methane load of 51.3 ± 2.2 g m^{-3} h^{-1}, was fed into R1 and R2 via three 10 μm porous stainless steel diffusers located at the bottom of the reactors. The stream was obtained by mixing a pure CH_4 stream (controlled by means of a mass flow controller, Aalborg, USA) with a pre-humidified air flow, resulting in a gas empty bed residence time (EBRT) of 30 min in the reactors. Both reactors were operated at 25 ºC and a pH of 9.0 ± 0.3, which was maintained via daily replacement of MSM at a dilution rate of 0.05 d^{-1}. The cultivation broth aliquots of 100 mL daily drawn from the reactors were used for the determination of the concentrations of biomass (measured as total suspended solids (TSS)), ectoine, hydroxyectoine, EPS and PHAs. Gas samples were also periodically taken using gas-tight syringes (HAMILTON, Australia) from the sampling ports located at the inlet and outlet of the bioreactors for the determination of CH_4 and CO_2 gas concentrations. The

elimination capacity (EC, g m^{-3} h^{-1}) and removal efficiency (RE, %) of the bioreactors were calculated as described by Nikiema et al. (2007)[22] Steady state operation was achieved when neither the EC nor RE deviated >10 % from the mean.

A mass transfer test was carried out under steady state at the end of C1, C2 and C3 in order to elucidate the limiting step during CH_4 biodegradation under the experimental conditions evaluated. For this purpose, the inlet CH_4 concentration was increased from 25.9 ± 2.1 g m^{-3} to 52.9 ± 3.6 g m^{-3} for a period of 6 h, and the concomitant variations in EC and CO_2 production were periodically recorded [23]. In this context, an increase in the inlet concentration of methane by a factor of 2 would result in 2 time higher CH_4 mass flow rates potentially available for the microbial community. Under a microbial activity limiting scenario, not enough bacteria would be available to degrade this additional methane load and therefore the EC would remain constant (similar to the EC before the increase in the CH_4 inlet load). However, if the process was limited by the mass transfer of CH_4 to the microbial community, the EC would eventually increase by a factor of 2 as a result of the higher CH_4 concentration gradient available for CH_4 mass transfer and the availability of a microbial community active enough to cope with this additional CH_4 load.

2.4 Analytical procedures

The intra-cellular ectoine and hydroxyectoine concentration was determined using 2 mL of cultivation broth according to Cantera et al. (2016b)[24]. The specific intra-cellular concentrations (g ectoine or g hydroxyectoine g biomass^{-1}) were calculated using the TSS concentration (g L^{-1}) of the corresponding cultivation broth, which was determined according to Standard methods[25]. The measurement was carried out by high performance liquid chromatography in a HPLC 717 plus auto-sampler (Waters, Bellefonte, USA) coupled with a UV Dual λ Absorbance detector (Waters, Bellefonte,

USA) at 210 nm and 40 ºC using a LC-18 AQ + C Supelcosil column (Waters, Bellefonte, USA) and a C18 AQ + pre-column (Waters, Bellefonte, EEUU). A phosphate buffer, consisting of 0.8 mM K_2HPO_4 and 6.0 mM Na_2HPO_4, was used as a mobile phase at 40 ºC and a flow rate of 1 mL min^{-1} [26]. Ectoine and hydroxyectoine quantification was carried out using external standards of commercially available ectoine and hydroxyectoine with a purity of 95 % (Sigma Aldrich, Spain).

PHAs analysis was carried out by centrifuging cultivation broth samples of 2 mL for 5 min at 9000 g and further processing the pellet according to López et al. (2014)[27]. The PHAs extracted from the samples were measured in a 7820A GC coupled with a 5977E MSD (Agilent Technologies, Santa Clara, USA) and equipped with a DB-wax column (30 m × 250 µm × 0.25 µm). The detector and injector temperatures were maintained at 250 ºC. The oven temperature was initially maintained at 40 ºC for 5 min, increased at 10 ºC min^{-1} up to 200 ºC and maintained at this temperature for 2 min. Finally, the oven temperature was increased up to 240 ºC at a rate of 5 ºC min^{-1}. The PHA content of the samples was referred to the volume of the corresponding cultivation broth used for analysis.

For EPS measurement, 0.5 mL of supernatant (after centrifugation for PHA analysis) were placed in a new dry and pre-weighted Eppendorf. The EPS fraction of the bacterial supernatant was precipitated by adding 1.5 mL of ethanol 90% (v/v) followed by an overnight incubation period. After incubation, the sample was centrifuged at 9000 g for 20 minutes and the supernatant was discarded. The sample was dried for 24 hours at 58 ºC and the mass of the precipitated EPS determined gravimentrically [28]. The EPS content of the samples was referred to the volume of the corresponding cultivation broth used for analysis.

Gas concentrations of CH_4 and CO_2 were determined in a Bruker 430 GC-TCD (Palo Alto, USA) equipped with a CP-Molsieve 5A (15 m × 0.53 µm × 15 µm) and a CP-PoraBOND Q (25 m × 0.53 µm × 10 µm) column. The oven, injector and detector temperatures were maintained at 45 ºC, 150 ºC and 200 ºC, respectively. Helium was used as the carrier gas at 13.7 mL min^{-1}.

2.6. Data analysis

The statistical data analysis was performed using SPSS 20.0 (IBM, USA). The results are given as the average ± standard deviation. The homogeneity of the variance of the parameters was evaluated using a Levene test. Significant differences were analysed by ANOVA and post-hoc analysis for multiple group comparisons. Differences were considered to be significant at $p \leq 0.05$.

2.7 Bacterial community analysis

Aliquots of 5 mL of the cultivation broths from the reactors under steady state were centrifuged at 9000 g for 10 min (Table 1). The resting pellet was used for DNA extraction with the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's instructions. DNA was quantified with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). The microbial composition of the 6 independently amplified reactor samples and mixed inoculum (Table 1) was characterized by sequencing two variable 16S rRNA gene regions, V4 and V5, in three separate sequencing runs on Illumina's HiSeq2000 platform using the 515f/926r primer pair according to Waters et al., 2017[29]. The microbial composition of the inoculum of R1 was not analysed since it was inoculated with a pure culture of *M. alcaliphilum* 20Z grown under sterile conditions. Illumina fastq files were de-multiplexed, quality filtered and analysed using QIIME (v. 1.9). For this purpose, different default settings and quality parameters were used according to Ramiro-García

et al. (2016) [30]. The 16S rRNA gene amplicons were filtered for quality (Q >25) and size (>200 bp), and phylogenetically classified[30]. The reference database used for taxonomic assignment was the SILVA database version 123 [31].

3. Results

3.1 Influence of Mg^{2+} concentration on ectoine production

The synthesis of intra-cellular ectoine under steady state was clearly influenced by Mg^{2+} concentration in both reactors. A Mg^{2+} concentration of 0.2 g L^{-1} was identified as the optimum value for the accumulation of intra-cellular ectoine, supporting average specific contents of 94.2 ± 10.1 and 79.7 ± 5.1 mg ectoine g $biomass^{-1}$ in R1 and R2, respectively. Lower Mg^{2+} concentrations supported lower ectoine yields: 51.0 ± 3.9 and 54.9 ± 3.5 mg ectoine g $biomass^{-1}$ in C2 and 35.0 ± 2.2 and 19.7 ± 0.8 mg ectoine g $biomass^{-1}$ in C3 in R1 and R2, respectively.

<Figure 1>

3.2 Influence of Mg^{2+} concentration on hydroxyectoine production

Mg^{2+} concentration also exerted a significant influence on the synthesis of this osmoprotectant (Figure 2). Mg^{2+} concentrations of 0.2 g L^{-1} mediated the accumulation of average concentrations of 13.3 ± 0.7 mg hydroxyectoine g $biomass^{-1}$ in R2, while lower Mg^{2+} concentrations supported lower hydroxyectoine yields in the same reactor: 9.05 ± 0.3 and 3.8 ± 0.1 mg hydroxyectoine g $biomass^{-1}$ in C2 and C3 respectively. Interestingly, hydroxyectoine was only detected in R1 at the lowest Mg^{2+} concentration (2.5 ± 0.2 mg hydroxyectoine g $biomass^{-1}$ in C3).

<Figure 2>

3.3 Influence of Mg^{2+} concentration on PHA production

PHAs were detected in the biomass present in both reactors at the three Mg^{2+} concentrations tested. The highest Mg^{2+} concentrations negatively impacted the accumulation of PHAs in R1 (1.3 ± 0.1 and 1.8 ± 0.1 mg PHA L^{-1} culture $broth^{-1}$ in C1 and C2, respectively), since biopolymers are generally produced under nutrient limiting conditions. Likewise, Mg^{2+} concentrations of 0.002 g L^{-1} induced the accumulation of PHAs up to average values of 14.6 ± 0.5 mg PHAs L^{-1} culture $broth^{-1}$ in R1. In the particular case of R2, Mg^{2+} concentration did not influence PHAs accumulation in the tested range (1.9 ± 0.1, 2.4 ± 0.1 and 2.4 ± 0.1 mg PHA L culture $broth^{-1}$ in C1, C2 and C3, respectively).

<Figure 3>

3.4 Influence of Mg^{2+} concentration on EPS production

EPS synthesis was also significantly influenced by Mg^{2+} concentration in both bioreactors. Hence, 0.2 g Mg^{2+} L^{-1} was identified as the optimum concentration for the synthesis of EPS, which reached yields of 1833 ± 87 and 2642 ± 83 mg EPS g $biomass^{-1}$ in R1 and R2, respectively (Figure 4). A decrease in Mg^{2+} concentration resulted in lower EPS yields: 1000 ± 124 and 757 ± 53 mg EPS g $biomass^{-1}$ in C2 and 952 ± 62 and 477 ± 47 mg EPS g $biomass^{-1}$ in C3 in R1 and R2, respectively.

<Figure 4>

3.5 Influence of Mg^{2+} concentration on CH_4 abatement performance

ECs of 16.1 ± 1.1, 18.9 ± 0.9 and 12.2 ± 0.3 g m^{-3} h^{-1} (corresponding to REs of 26.8 ± 1.8, 36.0 ± 1.9 and 20.8 ± 0.4 %) were recorded in R1 at 0.2, 0.02 and 0.002 g Mg^{2+} L^{-1}, respectively, while ECs of 16.2 ± 1.1, 15.05 ± 0.4 and 11.2 ± 0.7 g m^{-3} h^{-1} (corresponding to REs of 25.3 ± 1.1, 25.8 ± 0.6 and 18.5 ± 1.2 %) were recorded in R2 under similar Mg^{2+} concentrations (Figure 5). The mass transfer limitation tests conducted at the three Mg^{2+} concentrations tested revealed that an average increase in

the CH_4 inlet load of 2.2 resulted in a ×1.8 ± 0.12 increase in the EC in both reactors (Table S1. Supplementary Material). The fact that the higher CH_4 concentration gradient mediated by the increase in inlet CH_4 concentrations supported higher ECs in both bioreactors confirmed that process operation was limited by CH_4 mass transfer regardless the Mg^{2+} concentration tested [32]. Moreover, biomass concentrations did not show significant differences among the three Mg^{2+} concentrations tested in R1 (TSS concentrations of 1.2 ± 0.3, 1.7 ± 0.3, 1.7 ± 0.2 g L^{-1} in C1, C2 and C3, respectively) and R2 (TSS concentration of 1.3 ± 0.2, 1.1 ± 0.5, 1.7 ± 0.3 g L^{-1} in C1, C2 and C3, respectively).

<*Figure 5*>

3.6 Influence of Mg^{2+} concentration on the structure of bacterial population

Despite R1 was inoculated with a pure culture of *M. alcaliphilum* 20Z, the genus *Methylomicrobium* only represented 63 ± 2 % of the total bacterial population by the end of C1. At this stage, the genera *Methylophaga*, *Gelidibacter*, *Marinobacter* and *Halomonas* were also present in R1, with abundances of 12 ± 1 %, 5 ± 0, 5 ± 1 %, 2 ± 0 %, respectively (Figure 6). The reduction of Mg^{2+} concentration in the MSM to 0.02 Mg^{2+} L^{-1} gradually impacted on the population structure of R1 by the end of C2, *Methylophaga* (20 ± 2 %), *Gelidibacter* (18 ± 8 %), *Marinobacter* (5 ± 1 %), and *Halomonas* (4 ± 1 %) overcoming the genus *Methylomicrobium* to a 3 ± 1 % of the total bacterial population. Finally, the lowest Mg^{2+} concentration used in R1 mediated the dominance of the genera *Methylophaga*, *Gelidibacter*, *Marinobacter* and *Brevundimonas* with abundances of 32 ± 3, 12 ± 34, 8 ± 2, 8 ± 2 %, respectively.

<*Figure 6*>

The inoculum of R2, which was enriched from activated sludge, cow manure and soil, was composed mainly of *Methylomicrobium and Methylophaga* (41 ± 5 and 21 ± 4 %,

respectively). However, *Methylomicrobium* represented only 15 % of the total population by the end of the C1, value that remained constant until the end of experiment. *Methylophaga*, *Marinobacter* and *Halomonas*, which accounted for 16 ± 2, 25 ± 4, 26 ± 3 % of the total population by the end of C1 and C2, respectively, were able to compete better in this alkaline and saline environment than *Methylomicrobium*. The reduction in Mg^{2+} concentration resulted in a sharp increase of the dominance of a unknown uncultured family, previously isolated from mug, which represented 46 ± 2 % of the total bacterial community by the end of C3.

4. Discussion

4.1 Ectoines production

Magnesium exhibited a positive effect on the synthesis of ectoine regardless of the inoculum. Hence, 3 times higher ectoine yields were obtained at 0.2 mg Mg^{2+} L^{-1} compared to those recorded at 0.002 mg Mg^{2+} L^{-1}, likely due to the effect of Mg^{2+} on the ectoine biosynthesis pathway or on protein expression, transcription and translation. In this context, other environmental parameters such as NaCl, nitrogen, copper or the carbon source have been demonstrated to boost ectoine production when increasing their concentration[24,33,34]. Although the structure of bacterial population shifted as a function on Mg^{2+} concentration, most genera identified in R1 and R2 (i.e *Marinobacter*, *Methylophaga* or *Halomonas*) have been previously described as ectoine producers. For instance, the genus *Halomonas* can support ectoine accumulations of 150-170 mg g biomass^{-1}[8].

The ability of a given microorganism to synthesize hydroxyectoine inhererently depends on its ability to produce ectoine, since hydroxyectoine formation typically occurs directly from the conversion of either ectoine or one of its metabolic

intermediates[35]. In this context, Mg^{2+} could affect the ectoine production pathway without impacting on hydroxyectoine accumulation. It is noteworthy that hydroxyectoine was always detected in R2, but only present in R1 at low Mg^{2+} concentration. No hydroxyectoine accumulation has been reported to date in *M. alcaliphilum 20Z* cells grown at salinities up to 9 % NaCl[11]. This compatible solute is more common among gram-positive halophilic/halotolerant bacteria, although it is often co-synthesized with ectoine in a lower extent in many other ectoine-producing species (e.g. accumulations of 36.8 and 45 mg hydroxyectoine g $biomass^{-1}$ have been reported in *Halomonas boliviensis DSM 15516* and *Halomonas elongata KS3*, respectively)[33,36,37]. Therefore, the production of hydroxyectoine in both reactors was probably due to the presence of other bacterial population in both consortia, such as the genera *Halomonas, Methylophaga* and *Marinobacter*. In brief, the co-production of several ectoines during CH_4 abatement by a methanotrophic consortium composed of *Halomonas*, *Marinobacter*, *Methylomicrobium* and *Methylophaga* open up new opportunities for climate change mitigation.

4.2 Bioplastic production

Preliminary studies with methanotrophic bacteria suggested a positive effect of Mg^{2+} limitation on PHAs production. Thus, Wendland et al. (2001)[38] found PHAs contents of 28 % (g g $biomass^{-1}$) under Mg^{2+} limitation using a pure strain of *Methylocystys sp. GB25*, while PHAs contents of up to 50-60 % (g biomass) have been recently reported using *Methylocystis parvus OBBP* under a N and Mg^{2+} limitation [39,40]. However, the PHAs contents recorded in this study were much lower than the ones reported by other authors using *α-proteobacter* methanotrophs, since PHAs synthesis is supposed to be linked with the serine cycle. At this point it should be highlighted that despite nitrogen

limitation increases PHAs production[41], no nitrogen limitation was carried out in this study due to its negative effect on ectoine production, which has a higher profit margin than PHAs. Finally, the synthesis of PHA recorded during C3 in R1 could be directly attributed to the occurrence of the genera *Brevundimonas* and *Pannonibacter* under low Mg^{2+} concentrations, which are well known polyhydroxybutyrate producers[42,43].

4.3 Exopolysaccharides production

RuMP-pathway *γ-proteobacter* methanotrophs are able to support high productions of EPS. Thus, Malashenko et al. (2001) observed EPS yields ranging from 300 to 450 mg g $biomass^{-1}$ in *γ-proteobacter* methanotrophic cultures. In this context, the presence of *Methylomicrobium* and other *γ-proteobacteria* supports the high EPS yields obtained in this study. Moreover, previous studies have consistently demonstrated that stress conditions such as a high pH and high salinity can boost EPS biosynthesis [44,45]. Overall, the combination of a high alkalinity and salinity MSM with the occurrence of EPS producing microorganisms in the reactors resulted in EPS yields higher than those typically obtained in mesophilic methanotrophic cultures. However, the limitation in Mg^{2+} negatively affected the biosynthesis of exopolysaccharides.

4.4 Methane abatement performance

Significantly similar biomass concentrations were recorded in the reactors regardless of the Mg^{2+} concentration according to the TSS values. On the other hand, the mass transfer limitations tests carried out under steady state in C1, C2 and C3 demonstrated that both reactors were mass transfer limited despite the negative impact of Mg^{2+}

limitation on microbial biosynthesis metabolism. The decrease in EC recorded in both reactors under low Mg^{2+} limitation, which was not caused by a deterioration in the CH_4 biodegradation capacity of the community, was likely due to the biofouling of the gas diffusers in the reactor, which likely decreased the mass transfer capacity of the systems. The ECs and REs recorded in this study (ECs of XX-XX and YY-YY g m^{-3} h^{-1}, corresponding to REs of XX-XX and YY-YY % in R1 and R2, respectively) were in agreement with the ECs and REs reported in suspended-growth bioreactors operated with haloalkaliphilic methanotrophs, which typically range between 13.2 to 24.5 g m^{-3} h^{-1} (corresponding to REs of 22.5-31.3 %) [9,10]. However, these ECs and REs were lower than those obtained in conventional biofilters and biotrickling filters operated with mesophilic methanotrophs (ECs = 65-280 g m^{-3} h^{-1} corresponding to REs of 50-90 %)[46,47]. The lower elimination capacities here obtained were likely caused by both the detrimental effects of salt and alkalinity on methane solubility, which decrease methane mass transfer from the gas emission to the microorganisms, and the less effective metabolism of extremophiles microorganisms [48,49].

Finally, it is worth noting that most genera identified in the R1 and R2 have not been previously identified as methane degraders. In fact, only the genus *Methylomicrobium* is considered methanotrophic. Therefore, the fact that CH_4 ECs were maintained roughly similar during the complete experiment suggested that other microorganisms present in the reactors were able to use CH_4 as the only energy and carbon source.

5. Conclusion

This study fostered the potential of CH_4 biorefineries by validating the multi-production of high added market value metabolites from diluted CH_4 emissions. However, the

decrease in Mg^{2+} concentration from 0.2 to 0.002 mg L^{-1} did not significantly enhance PHAs but resulted in a severe deterioration in the biosynthesis of ectoines and exopolysaccharides. The identification of novel methanotrophic consortia able to co-produce ectoine and hydroxyectoine using methane as the sole feedstock, in addition to exopolysaccharides, can make methane abatement much more cost-effective and help mitigating climate change. In this context, the use of mixed methanotrophic communities in CH_4 biorefineries could provide more resilience and stability to the process while overcoming the current problems of metabolite productivity characteristic of *M. alcaliphilum 20Z*.

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