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Influence of operational conditions on the performance of biogas bioconversion into ectoines in pilot bubble column bioreactors

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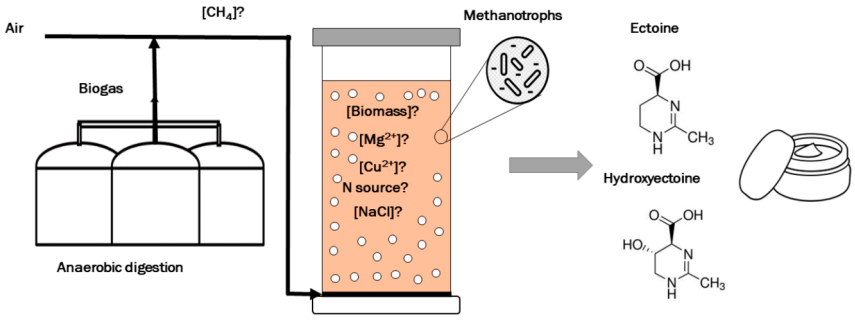
HIGHLIGHTS

- Low biomass concentrations resulted in an increase in the ectoines yields.
- The increase in Cu^{2+} and Mg^{2+} concentrations did not enhance ectoines synthesis.
- The use of ammonium instead of nitrate as N source induced biomass decay.
- The increase in CH_4 concentration did not enhance ectoines yields.
- Low NaCl concentrations enhanced CH_4 biodegradation but reduced ectoines yields.

GRAPHICAL ABSTRACT



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ABSTRACT

The application of biogas as a low-priced substrate for the production of ectoines constitutes an opportunity to decrease their production costs and to enhance the viability of anaerobic digestion. The influence of operational conditions on CH_4-biogas biodegradation and on ectoines production yields was assessed in continuous pilot bubble column bioreactors. The rise in biomass concentration from 1 to 3 g L^{-1} resulted in a decrease in the specific ectoine content from 42 ± 8 to 30 ± 4 $mg_{ectoine}$ g_{VSS}^{-1}. The concentration of Cu^{2+} and Mg^{2+} did not impact process performance, while the use of ammonium as N source resulted in low CH_4 biodegradation and ectoine yields (13 ± 7 $mg_{ectoine}$ g_{VSS}^{-1}). The increase in CH_4 content from 4.5 to 9 %v·v^{-1} enhanced CH_4 removal efficiency. Process operation at NaCl concentrations of 3 %w·w^{-1} instead of 6 %w·w^{-1} decreased the ectoine yield to 17 $mg_{ectoine}$ g_{VSS}^{-1}. Finally, *Methylomicrobium buryatense* was identified as the dominant species.

1. Introduction

The solute ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is highly valued in the cosmetic and pharmaceutical industries (Becker and Wittmann, 2020). The relevance of ectoine, with a market value of ~ 1000 € kg^{-1}, relies on its ability for the stabilization of DNA-protein complexes, enzymes and nucleic acids, which protect cells against hyper-osmotic stress and other extreme environments such as high temperature, drying and freezing (Pastor et al., 2010). Due to its outstanding properties, ectoine has become a highly-

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demanded product in the recent years. The industrial process for ectoine production, named "bacterial milking", is based on a concentrated cell cultivation of the pure species *Halomonas elongata* ATCC 33,173 in a high salinity medium, followed by a hypo-osmotic shock to release ectoine. However, this process exhibits high operational costs and environmental impacts due to the use of sterile conditions and costly carbon feedstock such as glucose or sodium glutamate (Sauer and Galinski, 1998; Van-Thuoc et al., 2010). On the other hand, its hydroxylated derivative, hydroxyectoine, presents similar properties to ectoine with an even superior protective effect against oxidative stress and extreme temperatures (Mustakhimov et al., 2019). However, despite hydroxyectoine can be synthesized by halophilic/halotolerant bacteria, its production at large scale has not been yet developed. In this context, the use of CH_4-biogas as a renewable feedstock for ectoines production constitutes an opportunity for decreasing its operational costs and minimizing its environmental impact (Pérez et al., 2021).

The capacity of halotolerant CH_4 oxidizing bacteria for the formation of ectoine and hydroxyectoine has been demonstrated beforehand (Cantera et al., 2017a; Khmelenina et al., 2000; Mustakhimov et al., 2019). Biogas constitutes a green energy vector traditionally used to produce heat and electricity due to its elevated CH_4 content (50–70 %) (Angelidaki et al., 2018). However, the rapid decrease in the prices of other renewable energy sources such as solar and wind power is jeopardizing the cost-competitiveness of electricity production from biogas (IRENA, 2020). Thereby, the generation of high value-added commodities from biogas such as ectoine and hydroxyectoine would enhance the economic sustainability of the anaerobic digestion of organic waste and might promote an expansion of the biogas industry (Pérez et al., 2021; Pieja et al., 2017).

Recent studies have evaluated the use of biogas for the biosynthesis of ectoine and hydroxyectoine. For instance, Cantera et al. (2020) recorded an ectoine concentration of 3–4 and 24–25 mg $g_{biomass}^{-1}$ using a diluted biogas stream with the pure culture *Methylomicrobium alcaliphilum* 20Z and a methanotrophic haloalkaliphilic consortium, respectively, at 6 %w·w^{-1} NaCl concentration in a 2 L bubble column bioreactor. In addition, Carmona-Martínez et al. (2021) reported ectoine and hydroxyectoine concentrations of 56.6 ± 2.5 and 51.0 ± 2.0 mg $g_{biomass}^{-1}$, respectively, using an enriched methanotrophic consortium at a NaCl concentration of 9%w·w^{-1}. However, the ectoine yields achieved to date in methanotrophic cultures are still low compared to those obtained in the industrial biomilking process (155 mg $g_{biomass}^{-1}$) and would require further process optimization (Sauer and Galinski, 1998). In this regard, Rodero et al. (unpublished) observed a more stable and efficient biogas bioconversion to ectoine in a single bubble column bioreactor operated at 6%w·w^{-1} NaCl compared to two bubble column bioreactors interconnected in series (at 0 and 6%w·w^{-1} NaCl, respectively). Interestingly, the maximum specific ectoine and hydroxyectoine concentrations (52 and 13 mg $g_{biomass}^{-1}$, respectively) were observed during the first week of operation, which suggested that biomass concentration could influence extremolites accumulation. In addition, the concentration of copper (Cu^{2+}), magnesium (Mg^{2+}) and NaCl in the cultivation broth has been proved to play an essential role on ectoine biosynthesis and/or on CH_4 fixation in batch methanotrophic cultures (Cantera et al., 2017a; Carmona-Martínez et al., 2021; Semrau et al., 2010). The nitrogen source has been also reported to impact ectoine production by *Halomonas salina* BCRC17875 using yeast extract and glutamate as a carbon source (Chen et al., 2018). Finally, Cantera et al. (2016b) observed an increase in ectoine yield from 14 to 31 mg $g_{biomass}^{-1}$ when increasing the content of CH_4 from 4 to 20 %v·v^{-1} during the batch cultivation of *Methylomicrobium alcaliphilum 20Z*. Unfortunately, the long-term influence of these environmental parameters on ectoine and hydroxyectoine biosynthesis and CH_4 uptake under continuous cultivation in high mass transfer bioreactors fed with air-biogas mixtures has not been carefully assessed.

In this work, the influence of the concentration of biomass, Cu^{2+} and Mg^{2+}, NaCl and inlet gas CH_4, and type of N source, on CH_4 biodegradation and ectoine and hydroxyectoine yields was investigated in flow-through bubble column bioreactors provided with an inner gas recirculation. In addition, the influence of the above mentioned environmental parameters on the structure of the methanotrophic population under long-term cultivation was investigated.

2. Materials and methods

2.1. Microorganisms and mineral salt medium

A bacterial methanotrophic consortium acclimated to 6%w·w^{-1} NaCl and previously enriched from a salt lagoon (Burgos, Spain) was used throughout the experiment (Carmona-Martínez et al., 2021). The mineral salt medium (MSM) contained (g L^{-1}): 3.78 $NaHCO_3$, 0.2 $MgSO_4{\cdot}7H_2O$, 0.013 $CaCl_2{\cdot}2H_2O$, 0.11 KH_2PO_4 and 0.13 $Na_2HPO_4{\cdot}2H_2O$, 26 μL of $Na_2WO_2{\cdot}2H_2O$ solution (2.7 g L^{-1}) and 2 mL of a trace elements solution prepared according to (Carmona-Martínez et al., 2021). KNO_3 or NH_4Cl were supplemented at a total N concentration of ~ 450 mg L^{-1}. NaCl concentration was 6 %w·w^{-1} unless specified otherwise.

2.2. Experimental set-up

The set-up comprised two 10 L bubble column bioreactors constructed with inner gas recirculation to boost CH_4 mass transfer. The bioreactors (67 cm height × 20 cm length × 10 cm width) made of clear PVC were interconnected to water condensers operated at 10 °C prior to the gas recirculation compressor to avoid operational problems due to water condensation (Fig. 1). A gas mixture comprised of synthetic biogas (70 %v·v^{-1} CH_4 and 30 %v·v^{-1} CO_2, Carburos Metalicos S.A. (Spain)) and air was continuously sparged into the bottom of the bioreactors through a custom-made membrane diffuser (0.5 mm pore size). The bioreactor was equipped with a 0.03 μm membrane module (Koch) in order to maintain the methanotrophic biomass inside the bioreactor during the MSM exchange. In this context, 1 L of biomass-free cultivation broth was replenished daily by fresh MSM to overcome nutrient limitation and inhibition by accumulation of toxic metabolites.

2.3. Operational stages and sampling procedures

Five sets of operating conditions were conducted to assess the influence of i) biomass concentration, ii) Cu^{2+} and Mg^{2+} concentration, iii) N source (NO_3^- *vs* NH_4^+), iv) CH_4 content in the inlet gas and v) NaCl concentration on methane degradation and ectoine/hydroxyectoine yields (Table 1). The two bioreactors were inoculated at the beginning of each operational stage. A biomass concentration of 3 g L^{-1} was set in the five operating stages, except for one of the bioreactors during stage 1, which was operated at a constant biomass concentration of 1 g L^{-1}. For this purpose, a fraction of the whole cultivation broth was periodically discarded based on the measurements of volatile suspended solids (VSS) concentration. To compensate the discarded broth volume, a similar volume of the biomass-free cultivation broth daily withdrawn was returned to the bioreactors. A gas inlet flowrate of ~ 0.23 L min^{-1}, which led to an empty bed residence time of 43.5 min, and an internal gas recirculation in the bioreactors of ~ 6.7 L min^{-1} (30 times the inlet gas flowrate) to improve the CH_4 mass transfer, were set during all operational stages. The inlet gas was composed of a *biogas:air* flowrate ratio of 1:13, which resulted in a CH_4 content of 5 %, excluding stage 4. In this stage, *biogas:air* flowrate ratios of 1:14.6 and 1:6.8 were used to reach a CH_4 contents of 4.5 and 9.0 %, respectively. The experiments were run at ambient temperature (≈ 22 °C) conditions and a stable pH of ≈ 8 (without control).

Gas samples of 100 μL were taken from the inlet and outlet gas streams three times per week with gas-tight syringes to measure gas concentrations (CH_4 and CO_2). Gas flowrates at the input and output streams of the bioreactors were also determined to accurately calculate

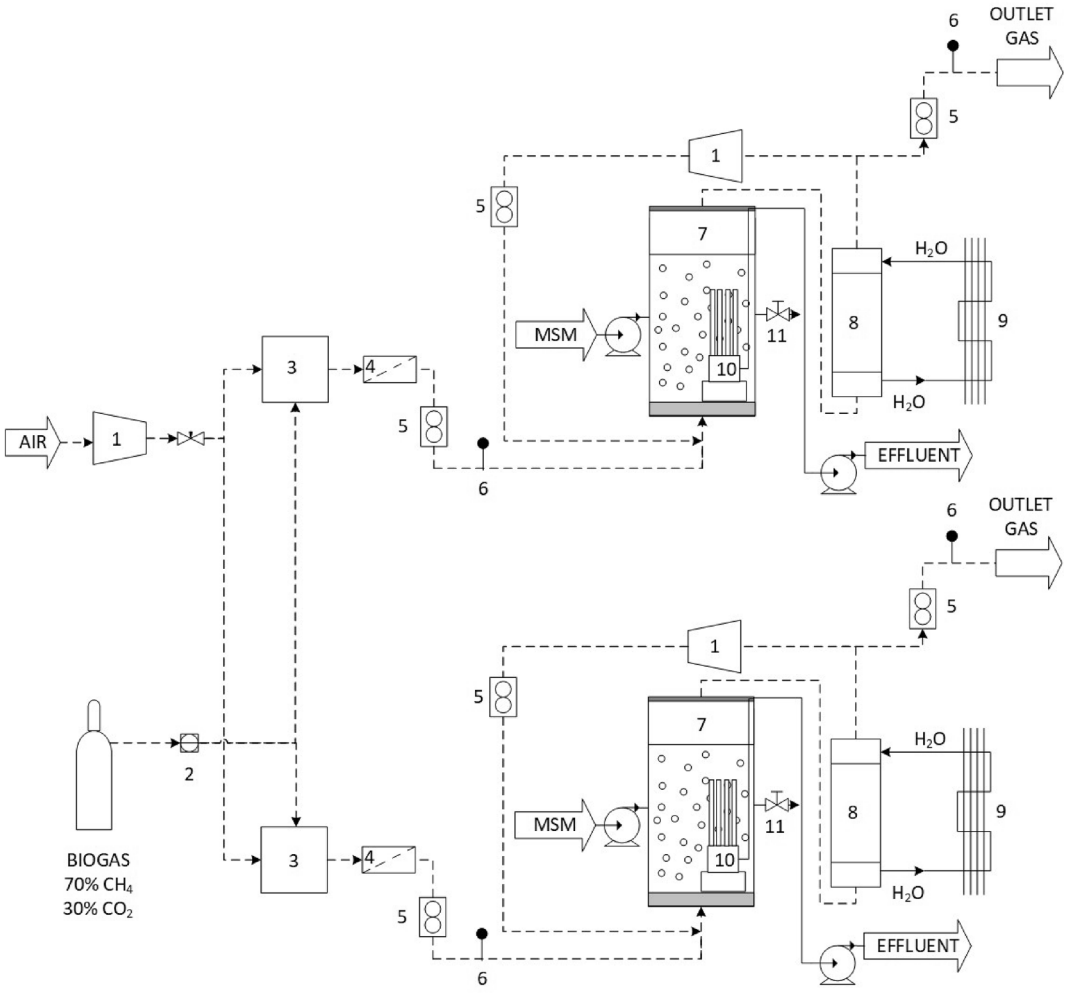

Fig. 1. Schematic diagram of the experimental set-up: (1) Compressor, (2) Mass flow controller, (3) Mixing chamber, (4) Gas filter, (5) Rotameter, (6) Gas sampling port, (7) Bubble column bioreactor, (8) Condenser, (9) Thermostatic bath, (10) Membrane module, (11) Liquid sampling port.

Table 1
Operational conditions tested during the optimization of biogas bioconversion into ectoines.

Stage	Duration (days)	Bioreactor	Biomass (g_{VSS} L^{-1})	[Cu^{2+}] (μM)	[Mg^{2+}] (mg L^{-1})	N source	[CH_4] (%v·v^{-1})	[NaCl] (%w·w^{-1})
1	31	1	1	0.5	20	KNO_3	5	6
		2	3	0.5	20	KNO_3	5	6
2	14	1	3	25	200	KNO_3	5	6
		2	3	0.5	20	KNO_3	5	6
3	23	1	3	0.5	20	NH_4Cl	5	6
		2	3	0.5	20	KNO_3	5	6
4	30	1	3	0.5	20	KNO_3	4.5	6
		2	3	0.5	20	KNO_3	9.0	6
5	30	1	3	0.5	20	KNO_3	5	3
		2	3	0.5	20	KNO_3	5	6

CH_4 removal efficiencies and CH_4 elimination capacities. Liquid samples of 200 mL from the cultivation broth were also drawn to determine pH, conductivity and concentration of dissolved total nitrogen (TN), dissolved organic carbon (TOC), dissolved inorganic carbon (IC), nitrate, nitrite, VSS, and intra-cellular ectoine and hydroxyectoine.

2.4. Analytical procedures

CH_4 and CO_2 gas concentrations were quantified using a Bruker 430 GC-TCD (Palo Alto, USA) integrated with a CP-Molsieve 5A and a CP-PoraBOND Q columns with helium as vector gas according to Cantera et al. (2020). The inlet gas pressure was monitored with a PN5007 Electronic pressure sensor (Ifm, Germany), while the inlet and outlet gas flowrates were controlled via rotameters (Aalborg) and confirmed by means of the water shifting method. Biomass concentration, reported as VSS, was measured according to standard methods (Eaton et al., 2005). The concentration of dissolved TOC, IC and TN was quantified following sample filtration using a 0.45 μm pore size filter using a Shimadzu TOC-

VCSH analyser (Japan) integrated with a TNM-1 chemiluminescence module. Nitrate and nitrite concentrations were determined by HPLC-IC according to Serejo et al. (2015). A Hach Sension + PH3 pH meter (Düsseldorf, Germany) was used for pH measurements, while conductivity was measured using an EC-Metro BASIC 30 instrument (Barcelona, Spain).

The intra-cellular ectoine and hydroxyectoine extraction procedure was conducted according to Rodero et al. (unpublished) prior determination via HPLC-UV (Cantera et al., 2020). The quantification of both components was performed with ectoine and hydroxyectoine (purity ≥ 95%, Sigma Aldrich) standards dissolved in ethanol at 70 %v·v^{-1}. The specific concentrations of ectoine and hydroxyectoine were estimated by dividing the above determined concentrations by the concentration of biomass (gVSS L^{-1}) in the bioreactors.

2.5. Bacterial community analysis

DNA isolation was conducted applying the protocol of the 'QIAamp PowerFecal Pro DNA kit' from Qiagen at ADM BIOPOLIS (Valencia, Spain). DNA purification and quality assessment, library preparation and sequencing were performed according to Carmona-Martínez et al. (2021). Then, raw sequences, forward and reverse, were imported into the QIIME2 platform (Bolyen et al., 2019). Cutadapt v3.4 was used to trim the forward and reverse adapters of the specific amplified region (Martin, 2011). Denoising, paired-ends joining and chimera removing were done using the DADA2 pipeline (Callahan et al., 2016). Those clean amplicon sequence variants were annotated against NCBI 16S rRNA database version 2021 using Blast version 2.2.29+ (Altschul et al., 1990) and against SILVA v.138 using NBAYES (Bokulich et al., 2018).

2.6. Determination of the CH_4 uptake, mass transfer performance and statistical analysis

CH_4 uptake was expressed in the form of CH_4 removal efficiency (CH_4-RE) and CH_4 elimination capacity (CH_4-EC) as follows:

$$CH_4 - RE\ (\%) = \frac{Q_{CH_{4,IN}} - Q_{CH_{4,OUT}}}{Q_{CH_{4,IN}}} \quad (1)$$

$$CH_4 - EC\ (gCH_4 m^{-3}\ h^{-1}) = \frac{Q_{IN} \times C_{CH_{4,IN}} - Q_{OUT} \times C_{CH_{4,OUT}}}{V} \quad (2)$$

where Q_{IN} and Q_{OUT} are the inlet and outlet gas flowrates (m^3 h^{-1}), c_{IN} and c_{OUT} the inlet and outlet methane concentration (g m^{-3}) and V (m^3) the working volume of the bioreactors.

The volumetric mass transfer coefficient of CH_4 (k_La_{CH4}) can be calculated using the CH_4-EC as follows:

$$k_La\ (h^{-1}) = \frac{CH_4 - EC}{\frac{C_{CH_{4,out}}}{H_{CH_4}} - C_{L,CH_4}} \quad (3)$$

where H_{CH4} is the dimensionless Henry's law constant (C_G/C_L) in water for CH_4 (29.1 at 22 °C) (Sander, 2015) and $C_{L,CH4}$ is the CH_4 concentration in the aqueous phase, which is negligible under limited CH_4 gas–liquid mass transfer. Although O_2 also exhibits a low solubility in water ($H_{O2} \approx 31.3$ at 22 °C), no limitation was encountered in this work due to its higher concentration in the inlet gas in the experiments. The k_La of O_2 was estimated from the O_2-EC as in the case of CH_4 at an inlet CH_4 content in the gas of 9 %v·v^{-1} to compare the mass transfer of CH_4 and O_2 under the most unfavourable O_2 mass transfer scenario.

As a result of CH_4 uptake, CO_2 and biomass are produced according to the following stoichiometric equations, depending on the N source:

$$CH_4 + 1.55\ O_2 + 0.07\ HNO_3 \rightarrow 0.71\ CO_2 + 0.07\ C_4H_8NO_2 + 1.75\ H_2O \quad (4)$$

$$CH_4 + 4.9\ O_2 + 0.2\ NH_4^+ \rightarrow CO_2 + 0.2\ C_5H_7NO_2 + 7.4\ H_2O \quad (5)$$

In addition, intermediate metabolites such as methanol or formaldehyde can be produced.

The volumetric CO_2 production rate (PCO_2) was calculated as:

$$PCO_2\ (g\ CO_2 m^{-3} h^{-1}) = \frac{Q_{OUT} \times C_{Co_{2,OUT}} - Q_{IN} \times C_{CO_{2,IN}}}{V} + \frac{Q_L \times (IC_{L,\ OUT} - IC_{L,\ IN})}{V} \times \frac{MW_{CO2}}{MW_C} \quad (6)$$

where Q_{IN} and Q_{OUT} are the inlet and outlet gas flow (m^3 h^{-1}), c_{IN} and c_{OUT} the inlet and outlet CO_2 mass concentration (g m^{-3}), V the working volume of the bioreactors (m^3), Q_L the flowrate of the cultivation broth replenished by fresh MSM (0.04 L h^{-1}), $IC_{L,\ in}$ and $IC_{L,\ out}$ the inlet and outlet inorganic carbon concentration in the mineral medium during the medium exchange (g L^{-1}) and MW the molecular weight of CO_2 and C.

A t-student statistical analysis with a significance $p \leq 0.05$ was performed in order to determine the effect of the different parameters studied on the CH_4 uptake and ectoines synthesis.

3. Results and discussion

3.1. Influence of the biomass concentration

Average CH_4-REs of 42.7 ± 5.4 and 47.3 ± 2.6 %, which corresponded with CH_4-ECs of 14.9 ± 2.2 and 16.9 ± 1.0 g CH_4 m^{-3}h^{-1}, were obtained under steady state at biomass concentrations of 1 and 3 g VSS L^{-1}, respectively (Fig. 2a,b). On the other hand, stationary state volumetric CO_2 production rates (PCO_2) of 12.3 ± 5.0 and 18.9 ± 4.7 g CO_2 m^{-3}h^{-1} were recorded at biomass concentrations of 1 and 3 g VSS L^{-1}, respectively (Fig. 2b). From these production rates, the 7 % and 10 % of the CO_2 produced at 1 and 3 g VSS L^{-1}, respectively, remained dissolved in the cultivation broth. These values corresponded to CH_4 mineralization ratios (PCO_2 / CH_4-EC) of 0.9 ± 0.4 and 1.0 ± 0.3 g CO_2 g^{-1} CH_4 at 1 and 3 g VSS L^{-1}, respectively, which were in the range typically reported for methanotrophic cultures (Acha et al., 2002; Rocha-Rios et al., 2011). In this regard, no significant difference on CH_4 removal regardless of the biomass concentration was observed (t-student test, $p > 0.05$), which suggested that biological CH_4 consumption was not the limiting factor on CH_4 removal under the experimental conditions tested. In this context, García-Pérez et al. (2018) reported that biomass concentrations >1 g VSS L^{-1} ensure that CH_4 gas–liquid mass transfer is the constraining step in bubble column bioreactors. Unfortunately, the CH_4-ECs herein obtained were lower than those previously reported (up to 74 g CH_4 m^{-3}h^{-1}) using *Methylocystis hirsuta* CSC1 at a biomass concentration of ~ 3.5 g L^{-1} with a similar internal gas recirculation (Rodríguez et al., 2020). It should be noted that Rodriguez et al. (2020) tested CH_4 inlet loads of up to 202 g CH_4 m^{-3}h^{-1} in a bubble column bioreactor constructed with 2 μm pore size diffusers. Process operation at lower CH_4 contents (5 %v·v^{-1} *vs* 14 %v·v^{-1}) and higher residence time (44 *vs* 30 min) in this study compared to Rodriguez et al. (2020) resulted in CH_4 inlet loads of ~ 35.5 g CH_4 m^{-3}h^{-1}, which could explain the superior CH_4-ECs obtained by Rodriguez et al. (2020).

A higher specific ectoine concentration was obtained at a biomass concentration of 1 g VSS L^{-1} (42 ± 8 $mg_{ectoine}\ g_{VSS}^{-1}$) compared to that at 3 g VSS L^{-1} (30 ± 4 $mg_{ectoine}\ g_{VSS}^{-1}$), which suggested a negative effect of increasing biomass concentrations on ectoine accumulation. Similarly,

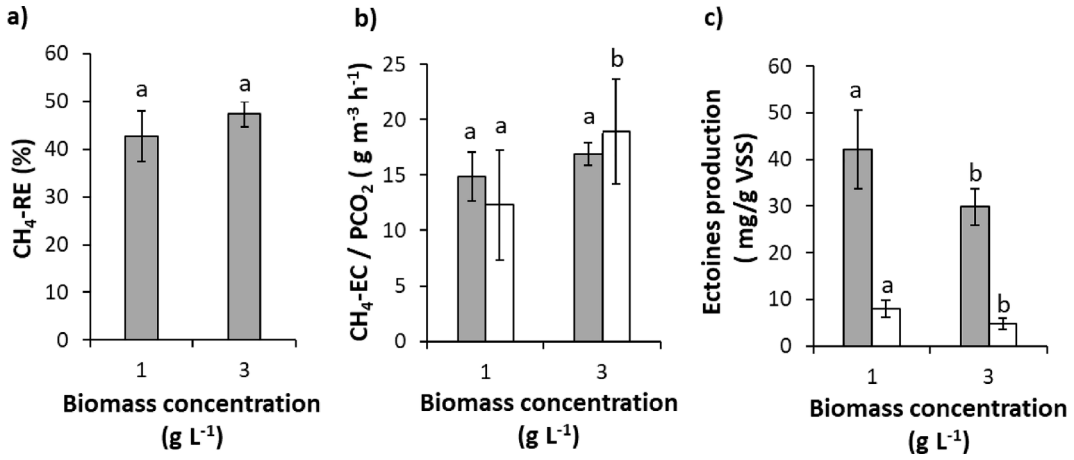

Fig. 2. Influence of the biomass concentration on a) CH_4 removal efficiency, b) CH_4 elimination capacity (grey) and volumetric CO_2 production rate (white) and c) specific ectoine (grey) and hydroxyectoine (white) concentration under steady state. Equal lowercase letters indicate no significant differences ($p > 0.05$) when comparing both biomass concentrations.

the specific hydroxyectoine content also decreased with the increase in biomass concentration (8 ± 8 and 4 ± 5 $mg_{hydroxyectoine}$ g_{VSS}^{-1} at biomass concentrations of 1 and 3 g VSS L^{-1}, respectively) (Fig. 2c). However, the increase in the specific ectoine and hydroxyectoine contents was not enough to compensate the use of biomass concentrations of 1 g VSS L^{-1} instead of 3 g VSS L^{-1}, and higher concentrations (80 *vs* 42 $mg_{ectoine}$ L^{-1} and 13 *vs* 8 $mg_{hydroxyectoine}$ L^{-1} at 3 and 1 g VSS L^{-1}, respectively) were obtained when operating at the highest. Net biomass productions of 0.07 and 0.12 g L^{-1} d^{-1} were obtained at the lowest and highest biomass concentrations. In this context, nitrogen concentration did not limit biomass growth or ectoine synthesis. Thus, TN concentrations of 330 ± 13 and 269 ± 26 mg N L^{-1} were recorded under steady state in the bioreactors operated at 1 and 3 g VSS L^{-1}, respectively. In agreement, Rodero et al. (unpublished) also observed a gradual decrease on the specific ectoine concentration from 52 to 27 $mg_{ectoine}$ g_{VSS}^{-1}, when the biomass concentration steadily increased from less than 1 g VSS L^{-1} up to 3 g VSS L^{-1} during the first weeks of operation in a 20 L bubble column bioreactor.

3.2. Influence of Cu^{2+} and Mg^{2+} concentration

The increase in Cu^{2+} and Mg^{2+} concentrations in the cultivation broth from 0.5 to 25 μM and from 20 to 200 mg L^{-1}, respectively, did not have a significant effect (t-student test, $p > 0.05$) on CH_4 biodegradation under steady state. Thus, average CH_4-REs and CH_4-ECs of 49.3 ± 3.1 % and 16.5 ± 0.6 g CH_4 $m^{-3}h^{-1}$, respectively, were reported under the lowest micronutrient concentrations and 53.6 ± 2.4 % and 18.1 ± 1.3 g CH_4 $m^{-3}h^{-1}$ under the highest Cu^{2+} and Mg^{2+} concentrations (Fig. 3a, b). In accordance, similar PCO_2 of 20.6 ± 5.2 and 19.8 ± 2.1 g CO_2 $m^{-3}h^{-1}$ (with associated CH_4 mineralization ratios of 1.2 ± 0.3 and 1.1 ± 0.1 g CO_2 g^{-1} CH_4) were recorded at the lowest and highest Cu^{2+} and Mg^{2+} concentration, respectively (Fig. 3b). In this context, approximately the 10% of the CO_2 produced remained dissolved in the cultivation broth in both bioreactors. Although previous works have reported superior CH_4 biodegradation rates with the increase in Cu^{2+} concentration in the cultivation broth in batch bioreactors (Cantera et al., 2016a; Ho et al., 2013), this effect was not significant in this study under continuous operation. Otherwise, no relevant effect of Mg^{2+} on CH_4 abatement was previously disclosed by Cantera et al. (2018) in methanotrophic culture composed of an haloalkaliphilic bacteria consortium.

Specific ectoine concentrations of 46 ± 2 and 36 ± 2 $mg_{ectoine}$ g_{VSS}^{-1} were recorded at the lowest and highest micronutrients concentrations, respectively. However, similar hydroxyectoine concentrations (6 ± 1 mg g_{VSS}^{-1}) were accumulated regardless of the Cu^{2+} and Mg^{2+} concentration (Fig. 3c). Surprisingly, the increase in the concentration of these micronutrients entailed a significant decrease in the intracellular ectoine concentration (t-student test, $p \leq 0.05$). Cantera et al. (2017a) reported no influence of the Cu^{2+} concentration on the intracellular ectoine accumulation in *Methylomicrobium alcaliphilum* 20Z. However, the increase in Cu^{2+} concentrations up to 25 μM promoted ectoine excretion in *Methylomicrobium alcaliphilum* 20Z. Cantera et al. (2017a) observed that the decrease in intracellular ectoine concentration could be attributed to

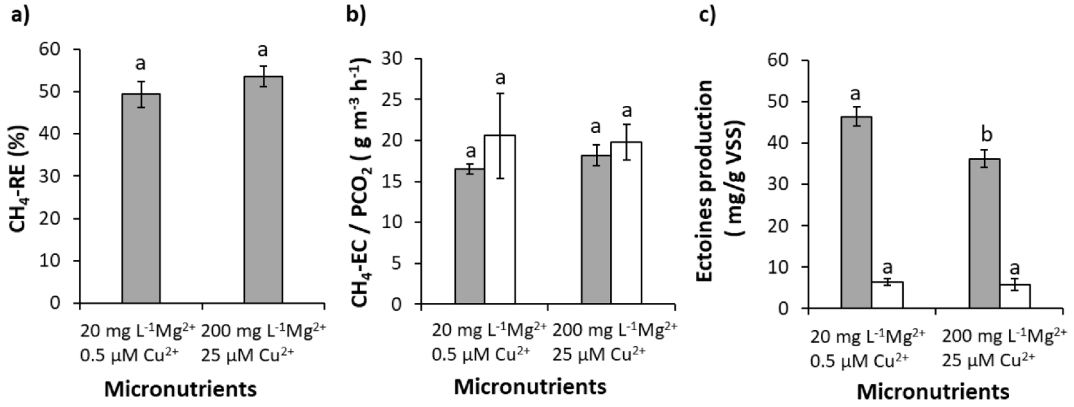

Fig. 3. Influence of the Cu^{2+} and Mg^{2+} concentration on a) CH_4 removal efficiency, b) CH_4 elimination capacity (grey) and volumetric CO_2 production rate (white) and c) specific ectoine (grey) and hydroxyectoine (white) concentration under steady state. Equal lowercase letters indicate no significant differences ($p > 0.05$) when comparing both Cu^{2+} and Mg^{2+} concentrations.

ectoine excretion due to the excess of Cu^{2+} concentration, which has been reported to repress the transcription of some proteins of the of S-layer in methanotrophs (Karlsen et al., 2010; Shchukin et al., 2011). Despite the positive effect of Mg^{2+} on the biosynthesis of ectoine reported in a previous study (Cantera et al., 2018), the increase in Mg^{2+} availability did not result in a higher intracellular ectoine concentration in this particular study.

3.3. Influence of the nitrogen source

The use of ammonium as a N source resulted in lower CH_4-REs (24.7 ± 2.9 %) compared to those obtained with nitrate (CH_4-REs of 51.5 ± 5.1 %), which corresponded with a reduction in CH_4-ECs from 18.0 ± 2.5 to 7.7 ± 1.3 g CH_4 $m^{-3}h^{-1}$ (Fig. 4a,b). The low performance of the methanotrophic consortium under operation with ammonium was attributed to either its competition for the enzyme MMO or the generation of nitrite (Hu and Lu, 2015). In this study, ammonium oxidation resulted in maximum nitrite concentration of 130 mg N-NO_2^- L^{-1}, which likely inhibited partially methanotrophic metabolism (see supplementary material). Despite the high O_2:CH_4 ratio hereby used (3.8:1), no oxidation of nitrite into nitrate was observed. Indeed, the biomass concentration set point (3 g VSS L^{-1}) was not reached in this bioreactor due to the low bacterial activity. On the other hand, the presence of high concentrations of O_2 avoided the reduction to nitrite when nitrate was applied as a N source in this study. As a result, the steady state PCO_2 recorded was also significantly lower (t-student test, $p \leq 0.05$) when using ammonium (7.2 ± 1.2 g CO_2 $m^{-3}h^{-1}$) than nitrate as a N source (21.6 ± 2.7 g CO_2 $m^{-3}h^{-1}$) (Fig. 4b). Indeed, process operation with ammonium resulted in a decrease in the IC concentration in the cultivation broth as a result of the nitrifying activity whereas it increased due to CO_2 dissolved (5 % of the total CO_2 produced) with the use nitrate. CH_4 mineralization ratios of 0.7 ± 0.2 g CO_2 g^{-1} CH_4 and 1.2 ± 0.2 g CO_2 g^{-1} CH_4 were reached in the presence of ammonium and nitrate, respectively. In this context, Rodríguez et al. (2020) reported a deterioration in methanotrophic activity due to the presence of nitrite at a maximum concentration of 83 mg N-NO_2^- L^{-1} as a result of the low O_2:CH_4 ratio in a bubble column bioreactor using nitrate as a N source. In addition, Rowe et al. (1979) claimed that nitrite has an inhibitory effect on oxygen uptake, ATP generation and active transport in the aerobic bacteria *Pseudomonas aeruginosa*.

The inhibition of the methanotrophic activity in the bioreactor operated with ammonium induced a lower ectoine accumulation in the culture broth (13 ± 7 $mg_{ectoine}$ g_{VSS}^{-1}) compared to the use of nitrate (44 ± 6 $mg_{ectoine}$ g_{VSS}^{-1}). Although hydroxyectoine is produced by a stereospecific hydroxylation of ectoine, similar hydroxyectoine yields (t-student test, $p > 0.05$) were recorded with both N sources (5 ± 1 and 6 ± 1 $mg_{hydroxyectoine}$ g_{VSS}^{-1} with ammonium and nitrate, respectively) (Fig. 4c). On the contrary, Chen et al. (2018) reported no negative effect of different ammonium salts at a concentration of 14 g L^{-1} on the growth of *Halomonas salina*, increasing ectoine production compared to other complex N sources. In this particular study, the use of a bacterial consortium instead of a pure culture, together with the higher availability of oxygen (which favored nitrite formation) and the use of CH_4 as a carbon source instead of organic carbon sources, could be the reason underlying the deterioration of microbial activity and, consequently, the decrease in ectoine production when ammonium was used as a N source.

3.4. Influence of the CH_4 content

Process operation at CH_4 contents of 4.5 and 9.0 %v·v^{-1} showed CH_4-REs of 44.5 ± 2.6 and 56.2 ± 1.7 %, with associated CH_4-ECs of 14.2 ± 1.2 and 31.8 ± 2.0 g CH_4 $m^{-3}h^{-1}$, respectively under steady state (Fig. 5a,b). The highest CH_4 removal was obtained at a CH_4 content of 9 %v·v^{-1}, which was attributed to the higher CH_4 gas–liquid concentration gradient. In this regard, the maximum CH_4 content that could be reached in the mixture biogas:air was 9 %v·v^{-1} to avoid O_2 limitation and comply with the minimum stoichiometric value required for the complete CH_4 oxidation. Although the k_La of the oxygen in the experiment conducted at a CH_4 content of 9 % v·v^{-1} (17.6 h^{-1}) was lower than that of CH_4 (28.7 h^{-1}), the higher gas–liquid concentration gradient resulted in O_2 mass transfer rates 3.2 times higher than those of CH_4 in this bioreactor. Likewise, a higher steady state PCO_2 (t-student test, $p \leq 0.05$) was obtained at 9 %v·v^{-1} CH_4 (22.1 ± 4.3 g CO_2 $m^{-3}h^{-1}$) compared to that recorded at 4.5 %v·v^{-1} CH_4 (15.5 ± 4.1 g CO_2 $m^{-3}h^{-1}$) (Fig. 5b). The increase in PCO_2 at CH_4 content of 9 %v·v^{-1} in the inlet gas entailed an increase in the proportion of the CO_2 produced that remained dissolved in the cultivation broth (14 % *vs* 7 % at 9 and 4.5 % v·v^{-1} CH_4, respectively). However, the increase in PCO_2 was not proportional to the increase in CH_4-EC, which entailed a decrease in the CH_4 mineralization ratio down to 0.7 ± 0.2 g CO_2 g^{-1} CH_4 at 9%v·v^{-1} CH_4 compared to that at 4.5 %v·v^{-1} CH_4 (1.1 ± 0.4 g CO_2 g^{-1} CH_4). In this context, Cantera et al. (2016b) also observed an increase in the specific CH_4 degradation rate from 0.33 to 1.50 g CH_4 h^{-1} $g_{biomass}^{-1}$ when CH_4 content was risen from 4 to 20 %v·v^{-1}.

Negligible influence of the inlet CH_4 content (t-student test, $p > 0.05$) on ectoine biosynthesis was observed in this study (Fig. 5c). Thus, specific ectoine concentrations of 41 ± 4 and 46 ± 4 mg g_{VSS}^{-1} were recorded at inlet CH_4 gas contents of 4.5 and 9 %v·v^{-1}, respectively. However, the specific hydroxyectoine concentrations slightly decreased from 8 ± 1 to 6 ± 1 mg g_{VSS}^{-1} with the increase of CH_4 content. On the contrary, Cantera

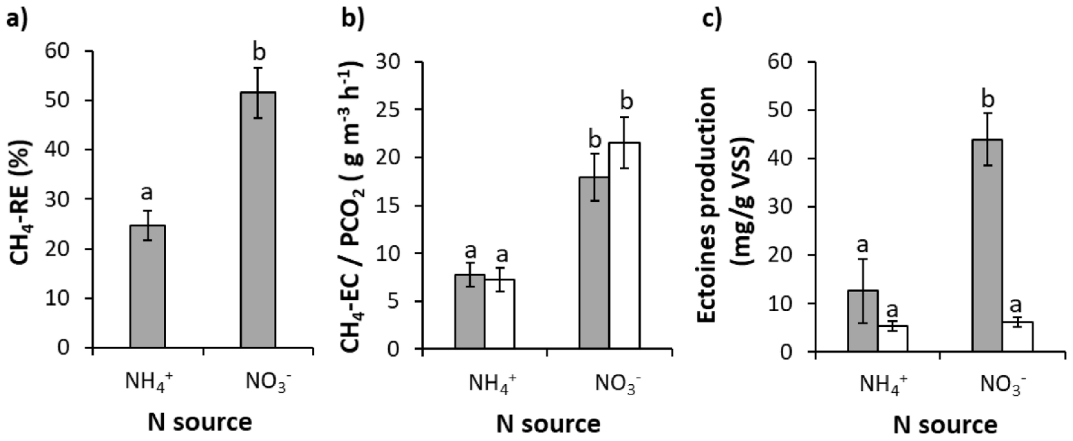

Fig. 4. Influence of the N source (NH_4^+ *vs* NO_3^-) on a) CH_4 removal efficiency, b) CH_4 elimination capacity (grey) and volumetric CO_2 production rate (white) and c) specific ectoine (grey) and hydroxyectoine (white) concentration under steady state. Equal lowercase letters indicate no significant differences ($p > 0.05$) when comparing both N sources.

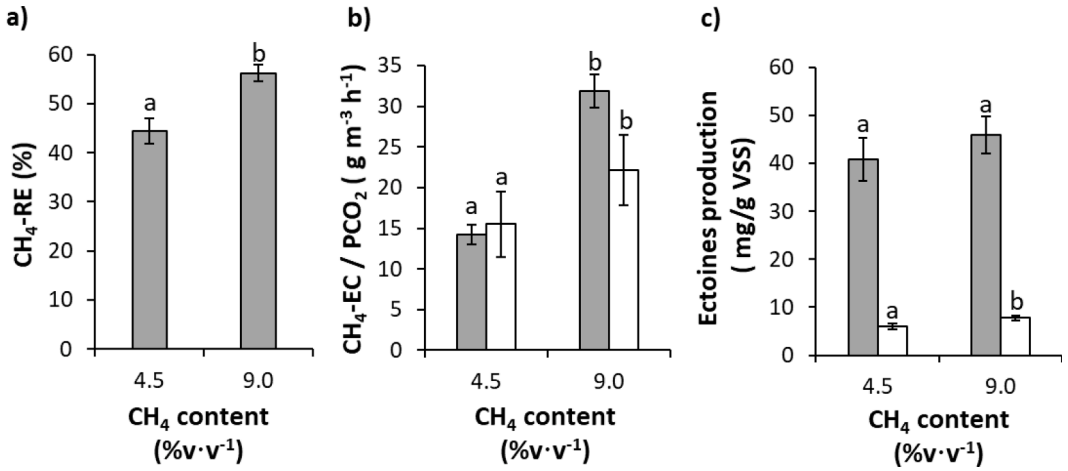

Fig. 5. Influence of the CH_4 content on a) CH_4 removal efficiency, b) CH_4 elimination capacity (grey) and volumetric CO_2 production rate (white) and c) specific ectoine (grey) and hydroxyectoine (white) concentration under steady state. Equal lowercase letters indicate no significant differences ($p > 0.05$) when comparing both CH_4 contents.

et al. (2016b) reported an increase in the maximum ectoine specific yields from 14 to 31 mg $g_{biomass}^{-1}$ when increasing the CH_4 content from 4 to 20 %v·v^{-1} in batch reactors under continuous magnetic agitation using *M. alcaliphilum* 20Z. The higher CH_4 gas content tested along with the lower biomass concentration in the batch tests likely supported a higher aqueous CH_4 content and the higher ectoine concentrations recorded in that study. In the present work, the results showed that a CH_4 content of 4.5 %v·v^{-1} was enough to avoid any limitation in the biosynthesis of ectoine and hydroxyectoine in the cultivation broth of the bubble column bioreactors.

3.5. Influence of the NaCl concentration

The decrease in NaCl concentration from 6 to 3 %w·w^{-1} in the cultivation broth, which corresponded to a decrease in the conductivity from 84.7 ± 1.0 to 51.0 ± 1.0 mS cm^{-1}, resulted in a slight increase (t-student test, $p \leq 0.05$) in the average CH_4-REs from 58.4 ± 3.0 to 66.5 ± 2.2 % under steady state (Fig. 6a). Similarly, slightly lower CH_4-ECs of 20.5 ± 1.6 g CH_4 $m^{-3}h^{-1}$ were recorded at 6 %w·w^{-1} compared to the CH_4-ECs of 22.2 ± 1.5 g CH_4 $m^{-3}h^{-1}$ at 3 %w·w^{-1} NaCl (Fig. 6b). This difference in CH_4 removal performance could be explained by a decrease in the CH_4 gas–liquid mass transfer (CH_4-k_La of 47.1 *vs* 36.8 h^{-1} at 3 and 6 % w·w^{-1} NaCl, respectively) due to the increase in NaCl concentration, which resulted in a lower CH_4 aqueous solubility (Wiesenburg and Guinasso, 1979). Interestingly, no negative effect of the increase in NaCl concentration in the range 3–6 %w·w^{-1} on biomass growth (maintained at 3 g SSV L^{-1}) was observed in the present study, which could be attributed to the use of a culture previously acclimated to 6 %w·w^{-1} NaCl. Indeed, similar average PCO_2 (27.5 ± 5.8 and 27.4 ± 2.6 g CO_2 $m^{-3}h^{-1}$), of which ≈7 % remained dissolved in the cultivation broth, and CH_4 mineralization ratios (1.2 ± 0.1 and 1.4 ± 0.4 g CO_2 g^{-1} CH_4 at NaCl concentrations of 3 and 6 %w·w^{-1}, respectively) were obtained, which supported a similar bacterial activity in both bioreactors (Fig. 6b). Likewise, Cantera et al. (2017b) reported no differences on PCO_2 (14 g CO_2 $m^{-3}h^{-1}$) and CH_4 mineralization ratios (1.1–1.2 g CO_2 g^{-1} CH_4) at 0 and 6 %w·w^{-1} NaCl using *M. alcaliphilum* 20Z. On the contrary, Carmona-Martínez et al. (2021) observed a decrease in the CH_4 biodegradation rates from 0.92 to 0.56 g CH_4 $m^{-3}h^{-1}$ using an enriched methanotrophic consortium from salt lagoon in a batch study conducted at 3 and 6 %w·w^{-1}, respectively. This decrease was attributed to the deterioration in microbial activity resulted from the high salinity conditions. Similarly, Cantera et al. (2016b) reported a decrease in the specific CH_4 degradation rate from 0.34 to 0.22 g CH_4 h^{-1} $g_{biomass}^{-1}$ when the NaCl concentration was raised from 3 to 6 %w·w^{-1} using *Methylomicrobium alcaliphilum* 20Z.

A significant decrease in ectoine and hydroxyectoine contents (t-

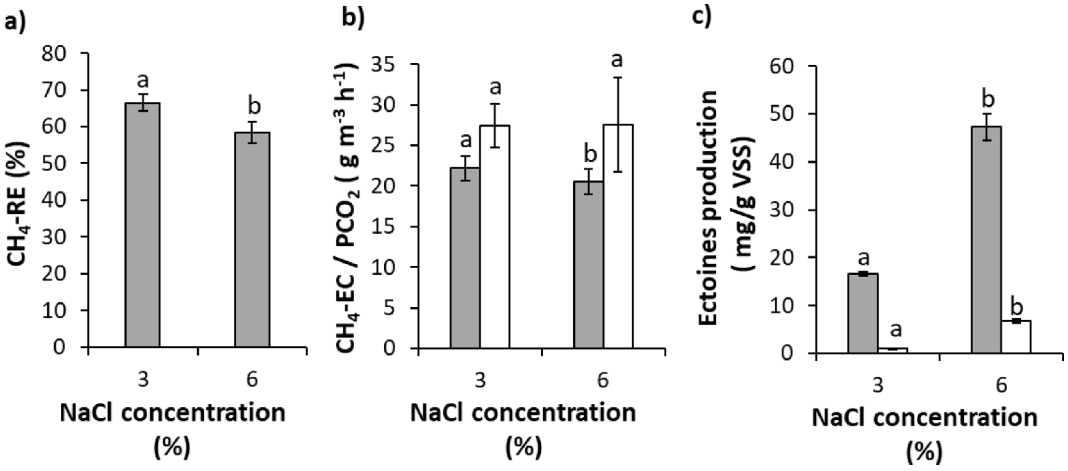

Fig. 6. Influence of the NaCl concentration on a) CH_4 removal efficiency, b) CH_4 elimination capacity (grey) and volumetric CO_2 production rate (white) and c) specific ectoine (grey) and hydroxyectoine (white) concentration under steady state. Equal lowercase letters indicate no significant differences ($p > 0.05$) when comparing both NaCl concentrations.

student test, $p \leq 0.05$) was observed at decreasing NaCl concentrations (Fig. 6c). Thus, 47 ± 3 $mg_{ectoine}\ g_{VSS}^{-1}$ and 7 ± 0 $mg_{hydroxyectoine}\ g_{VSS}^{-1}$ and 17 ± 0 $mg_{ectoine}\ g_{VSS}^{-1}$ and 1 ± 0 $mg_{hydroxyectoine}\ g_{VSS}^{-1}$ were obtained at NaCl concentrations of 6 and 3 %w·w^{-1}, respectively. In this context, since these extremolites are accumulated as a response of salt stress by halotolerant methanotrophs, a direct correlation between NaCl concentration and their production was expected. Similarly, But et al. (2013) reported a ~ 2-fold increase in the ectoine accumulation (from 14 to 28 mg $g_{biomass}^{-1}$) in *M. alcaliphilum* 20Z with the increase in the NaCl concentration from 3 to 7 %w·w^{-1}. In addition, Cantera et al. (2016b) obtained the highest ectoine yield at a NaCl concentration of 6 %w·w^{-1} (67 $mg_{ectoine}\ g_{biomass}^{-1}$), ectoine concentrations decreasing down to 30 mg $g_{biomass}^{-1}$ at 9 %w·w^{-1} of NaCl.

3.6. Microbial communities

Both bioreactors were at the beginning inoculated with a consortium of bacteria containing the following main genera: *Nitratireductor* (36 %), *Aequorivita* (13 %), *Methylomicrobium* (8 %), *Methylophaga* (8 %), *Stappia* (5 %), *Planktosalinus* (5 %), *Blastopirellula* (4 %), *Halomonas* (3 %), *Cryomorpha* (3 %), *Hyphomonas* (2 %) and *Alteromonadales* (2 %) (Rodero et al., n.d.). At the end of the first stage, the genus *Methylomicrobium* (36.1–36.7 %), which is able to accumulate ectoine using CH_4 as the unique energy and carbon input, was the most abundant, while the share of the genus *Nitratireductor* decreased down to 2.2–4.6 %, regardless the biomass concentration (see supplementary material). Interestingly, *Methylomicrobium japanense* (20.3 %) and *Methylomicrobium buryatense* (16.4 %) were present in the bioreactor operating at biomass concentration of 1 g VSS L^{-1}, whereas only *M. buryatense* was detected with a relative abundance value ≥ 1 % at a biomass concentration of 3 g VSS L^{-1}. *Aequorivita viscosa* (12.6–12.7 %) and *Methylophaga nitratireducenticrescens* (8.6–10.3 %) also exhibited a high relative abundance under steady state in both bioreactors. The presence of dissolved organic matter from CH_4 biodegradation in the cultivation broth along with the environmental conditions in both bioreactors (aerobic conditions, pH of 8.0 ± 0.1, temperature of ~ 22 °C and 6 % w·w^{-1} NaCl) promoted the growth of the non-methanotrophic species *A. viscosa* (Liu et al., 2013). Interestingly, the high concentration of nitrate and the environmental conditions in the cultivation broth were also favorable for the proliferation of *M. nitratireducenticrescens* (Villeneuve et al., 2013). Although ectoine or hydroxyectoine production has not been previously verified in these bacteria, other strains from the *Methylophaga* genus have been previously recognized as producers of ectoine (Antony et al., 2012; Cantera et al., 2020; Shmareva et al., 2018). In addition, *Halomonas alimentaria* was also detected in both bioreactors with a higher abundance in the bioreactor operating at 1 g VSS L^{-1} (6.6 %) compared with the bioreactor at 3 g VSS L^{-1} (2.9 %). In this regard, the *Halomonas* genus has been previously described as a potential ectoine and hydroxyectoine producer (Pastor et al., 2010). Overall, a more efficient ectoine synthesizing consortium could have contributed to the superior ectoine and hydroxyectoine contents in the bioreactor operated at 1 g VSS L^{-1}.

Methylomicrobium was the main genus in both bioreactors at the end of the second stage. It is noteworthy that *M. buryatense* was the only specie from the genus *Methylomicrobium* with a relative abundance ≥ 1% since both bioreactors were inoculated with the bacterial biomass from the bioreactor operating at 3 g VSS L^{-1} in the previous stage. However, the abundance of *M. buryatense* differed considerably between both bioreactors, being 45.1 % in the bioreactor with the lowest concentration of Cu^{2+} and Mg^{2+} and 30.2 % in the bioreactor with the highest concentration of micronutrients. In this context, Cantera et al. (2016a) reported a higher abundance of the *Methylomicrobium* genus in enrichments conducted at a Cu^{2+} concentration of 0.05 μM compared to those at 25 μM Cu^{2+}. On the other hand, the increase in the availability of these micronutrients entailed a significant increase in the share of the species *Aequorivita lipolytica* up to 18.0 %. This microorganism is able to tolerate saline concentrations up to 6 %w·w^{-1} NaCl (Bowman and Nichols, 2002). However, no connection of this species with methanotrophic metabolism or ectoine production has been reported up to date. Furthermore, the addition of Cu^{2+} and Mg^{2+} also favored the growth of the species *Nitratireductor aquibiodomus* and *N. aquimarinus,* which increased up to 9.5 %. *N. aquibiodomus* strain, which has the ability to reduce nitrate to nitrite, is capable to synthesize ectoine under osmotic stress conditions (Singh et al., 2012). Other genera such as *Methylophaga nitratireducenticrescens* (7.9–8.9 %) and *Aequorivita viscosa* (6.2–6.8 %) were also present in a high relative abundance in both bioreactors. Finally, it should be noted that the fact that ectoine synthesis was lower in the bioreactor operated at a higher concentration of Cu^{2+} and Mg^{2+} could be associated to a decrease in the abundance of microorganisms able to produce ectoine.

The use of ammonium as a N source instead of nitrate in the MSM impacted on the population structure in the bioreactor, with *Aequorivita viscosa* (23.6%) overcoming *Methylomicrobium buryatense* (19.1 %) by the end of stage 3. The increase in the concentration of extracellular organic matter in the medium caused by cell decay and the presence of N in form of ammonium could have mediated the increase in *A. viscosa* population. Surprisingly, *Methylophaga nitratireducenticrescens* and the genus *Nitratireductor* were also present at a high abundance in the bioreactor operating with ammonium. On the other hand, the population structure in the bioreactor operated with nitrate was similar to that of the inoculum, which was obtained from the previous stage at a Cu^{2+} and Mg^{2+} concentration of 0.5 μM and 20 mg L^{-1}, respectively.

The inlet CH_4 content partially impacted on the structure of the microbial population in the bioreactors during stage 4. Thus, despite *Methylomicrobium buryatense* was the most abundant species in both bioreactors, with a share of 39.4 and 34.4 % at CH_4 contents of 4.5 % v·v^{-1} and 9.0 %v·v^{-1}, respectively, process operation with 9 %v·v^{-1} CH_4 entailed a remarkable increase in the genus *Nitratireductor* up to 18.8 %. The lower availability of O_2 due to the decrease in the O_2:CH_4 ratio (from 4.3 to 2.1), and the high concentration of nitrate in the cultivation broth could have supported a higher metabolic activity of these nitrate reducers (Jang et al., 2011; Labbé et al., 2004). In this context, the increase in the abundance of *N. aquibiodomus* and *N. aquimarinus* did not exert a negative impact on ectoine production since these microorganisms harbor the genes responsible for ectoine biosynthesis (Singh et al., 2012). Other abundant microbial species detected in both bioreactors were *Methylophaga nitratireducenticrescens* (9.3–10.1 %) and *Lacimicrobium alkaliphilum* (7.6–10.3 %). Despite *L. alkaliphilum* has not been previously identified as a methanotrophic or ectoine producing bacteria, the environmental conditions in the cultivation broth were optimum for its development (Zhong et al., 2016).

The decrease in NaCl concentration from 6 to 3 %w·w^{-1} influenced the relative abundance of the major genera present in the bioreactors under steady state in stage 5. The *Nitratireductor* genus increased up to 30.6 %, overcoming the specie *Methylomicrobium buryatense* whose abundance dropped to a 16.8 % of the total bacterial population in the bioreactor operated at NaCl concentration of 3 %w·w^{-1}, whereas *M. buryatense* (41.7 %) represented the most abundant species in the bioreactor operated at NaCl 6 %w·w^{-1}. The decrease in NaCl concentration also enhanced the growth *N. aquibiodomus* or/and *N. aquimarinus,* the latter exhibiting an optimum growth under 3–4 % w·w^{-1} NaCl (Jang et al., 2011). Interestingly, the decrease in the abundance of the methanotroph *M. buryatense* in the bioreactor operated at 3 %w·w^{-1} NaCl did not exert a negative impact on CH_4 biodegradation. *Methylophaga nitratireducenticrescens* was also present at high relative abundances (14.8–15.2 %) in both bioreactors. In addition, the decrease in NaCl concentration down to 3 %w·w^{-1} increased the presence of the non-methanotroph *Stappia indica* (11.9 %) in this bioreactor (Lai et al., 2010), while *Lacimicrobium alkaliphilum* maintained a high abundance in the bioreactor at 6 %w·w^{-1} NaCl (9.2 %). Unfortunately, no data about the production of ectoine has been reported in these species up to date. However, since the main genera detected in both

bioreactors (*Methylomicrobium, Nitratireductor* and *Methylophaga*) are known as ectoine producers, the decrease in ectoines yields at 3 %w·w^{-1} NaCl was mainly attributed to the decrease of the salinity stress.

4. Conclusions

This study provided novel insights into the optimal operational conditions supporting maximum ectoines production from biogas. Lower biomass concentrations showed a higher specific accumulation of ectoines but a reduced overall ectoine productivity. The increase in Cu^{2+}, Mg^{2+} concentrations and CH_4 content did not enhance ectoine production yields. The use of ammonium instead of nitrate as a nitrogen source was detrimental to methanotrophic activity. The decrease in NaCl content enhanced CH_4 biodegradation at the expenses of lower ectoines yields. Finally, *Methylomicrobium buryatense* was herein identified as the dominant species responsible of bioconverting biogas into ectoines regardless of the operational conditions.

E-supplementary data for this work can be found in e-version of this paper online.

CRediT authorship contribution statement

María del Rosario Rodero: Investigation, Formal analysis, Visualization, Writing – original draft. **Raquel Herrero-Lobo:** Investigation. **Víctor Pérez:** Formal analysis, Writing – review & editing. **Raúl Muñoz:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.127398.

References

Acha, V., Alba, J., Thalasso, F., 2002. The absolute requirement for carbon dioxide for aerobic methane oxidation by a methanotrophic-heterotrophic soil community of bacteria. Biotechnol. Lett. 24, 675–679. https://doi.org/10.1023/A:1015265530501.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., Kougias, P.G., 2018. Biogas upgrading and utilization: Current status and perspectives. Biotechnol. Adv. 36, 452–466. https://doi.org/10.1016/j.biotechadv.2018.01.011.

Antony, C.P., Doronina, N.V., Boden, R., Trotsenko, Y.A., Shouche, Y.S., Colin Murrell, J., 2012. Methylophaga lonarensis sp. nov., a moderately haloalkaliphilic methylotroph isolated from the soda lake sediments of a meteorite impact crater. Int. J. Syst. Evol. Microbiol. 62, 1613–1618. https://doi.org/10.1099/ijs.0.035089-0.

Becker, J., Wittmann, C., 2020. Microbial production of extremolytes — high-value active ingredients for nutrition, health care, and well-being. Curr. Opin. Biotechnol. 65, 118–128. https://doi.org/10.1016/j.copbio.2020.02.010.

Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A., Gregory Caporaso, J., 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6, 90. https://doi.org/10.1186/s40168-018-0470-z.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K. C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37 (8), 852–857.

Bowman, J.P., Nichols, D.S., 2002. Aequorivita gen. nov., a member of the family Flavobacteriaceae isolated from terrestrial and marine Antarctic habitats. Int. J. Syst. Evol. Microbiol. 52, 1533–1541. https://doi.org/10.1099/ijs.0.01976-0.

But, S.Y., Khmelenina, V.N., Mustakhimov, I.I., Trotsenko, Y.A., 2013. Construction and characterization of Methylomicrobium alcaliphilum 20Z knockout mutants defective in sucrose and ectoine biosynthesis genes. Microbiol. (Russian Fed. 82, 253–255. https://doi.org/10.1134/S0026261713020021.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High resolution sample inference from Illumina amplicon data. Nat Methods 13, 1–7. https://doi.org/10.1038/nmeth.3869.

Cantera, S., Lebrero, R., García-Encina, P.A., Muñoz, R., 2016a. Evaluation of the influence of methane and copper concentration and methane mass transport on the community structure and biodegradation kinetics of methanotrophic cultures. J. Environ. Manage. 171, 11–20. https://doi.org/10.1016/j.jenvman.2016.02.002.

Cantera, S., Lebrero, R., Rodríguez, E., García-Encina, P.A., Muñoz, R., 2017a. Continuous abatement of methane coupled with ectoine production by Methylomicrobium alcaliphilum 20Z in stirred tank reactors: A step further towards greenhouse gas biorefineries. J. Clean. Prod. 152, 134–141. https://doi.org/10.1016/j.jclepro.2017.03.123.

Cantera, S., Lebrero, R., Rodríguez, S., García-Encina, P.A., Muñoz, R., 2017b. Ectoine bio-milking in methanotrophs: A step further towards methane-based bio-refineries into high added-value products. Chem. Eng. J. 328, 44–48. https://doi.org/10.1016/j.cej.2017.07.027.

Cantera, S., Lebrero, R., Sadornil, L., García-Encina, P.A., Muñoz, R., 2016b. Valorization of CH_4 emissions into high-added-value products: Assessing the production of ectoine coupled with CH_4 abatement. J. Environ. Manage. 182, 160–165. https://doi.org/10.1016/j.jenvman.2016.07.064.

Cantera, S., Phandanouvong-Lozano, V., Pascual, C., García-Encina, P.A., Lebrero, R., Hay, A., Muñoz, R., 2020. A systematic comparison of ectoine production from upgraded biogas using Methylomicrobium alcaliphilum and a mixed haloalkaliphilic consortium. Waste Manag. 102, 773–781. https://doi.org/10.1016/j.wasman.2019.11.043.

Cantera, S., Sánchez-Andrea, I., Lebrero, R., García-Encina, P.A., Stams, A.J.M., Muñoz, R., 2018. Multi-production of high added market value metabolites from diluted methane emissions via methanotrophic extremophiles. Bioresour. Technol. 267, 401–407. https://doi.org/10.1016/j.biortech.2018.07.057.

Carmona-Martínez, A.A., Marcos-Rodrigo, E., Bordel, S., Marín, D., Herrero-Lobo, R., García-Encina, P.A., Muñoz, R., 2021. Elucidating the key environmental parameters during the production of ectoines from biogas by mixed methanotrophic consortia. J. Environ. Manage. 298, 113462.

Chen, W.C., Hsu, C.C., Lan, J.C.W., Chang, Y.K., Wang, L.F., Wei, Y.H., 2018. Production and characterization of ectoine using a moderately halophilic strain Halomonas salina BCRC17875. J. Biosci. Bioeng. 125, 578–584. https://doi.org/10.1016/j.jbiosc.2017.12.011.

Eaton, M.A.H., Clesceri, A.D., Rice, L.S., Greenberg, E.W., Franson, A.E., 2005. APHA: Standard Methods for the Examination of Water and Wastewater, Centen. ed. APHA, AWWA, WEF, Washington, DC.

García-Pérez, T., López, J.C., Passos, F., Lebrero, R., Revah, S., Muñoz, R., 2018. Simultaneous methane abatement and PHB production by Methylocystis hirsuta in a novel gas-recycling bubble column bioreactor. Chem. Eng. J. 334, 691–697. https://doi.org/10.1016/j.cej.2017.10.106.

Ho, A., Lüke, C., Reim, A., Frenzel, P., 2013. Selective stimulation in a natural community of methane oxidizing bacteria: Effects of copper on pmoA transcription and activity. Soil Biol. Biochem. 65, 211–216. https://doi.org/10.1016/j.soilbio.2013.05.027.

Hu, A., Lu, Y., 2015. The differential effects of ammonium and nitrate on methanotrophs in rice field soil. Soil Biol. Biochem. 85, 31–38. https://doi.org/10.1016/j.soilbio.2015.02.033.

IRENA, 2020. Renewable Power Generation Costs in 2020, Renewable Power Generation Costs in 2020.

Jang, G.I.I., Hwang, C.Y., Cho, B.C., 2011. Nitratireductor aquimarinus sp. nov., isolated from a culture of the diatom Skeletonema costatum, and emended description of the

genus Nitratireductor. Int. J. Syst. Evol. Microbiol. 61, 2676–2681. https://doi.org/10.1099/ijs.0.028373-0.
Karlsen, O.A., Larsen, J., H.b.,, 2010. Identification of a bacterial di-haem cytochrome c peroxidase from Methylomicrobium album BG8. Microbiology 156, 2682–2690. https://doi.org/10.1099/mic.0.037119-0.
Khmelenina, V.N., Sakharovskii, V.G., Reshetnikov, A.S., Trotsenko, Y.A., 2000. Synthesis of osmoprotectants by halophilic and alkaliphilic methanotrophs. Mikrobiologiya 69 (4), 381–386.
Labbé, N., Parent, S., Villemur, R., 2004. Nitratireductor aquibiodomus gen. nov., sp. nov., a novel α-proteobacterium from the marine denitrification system of the Montreal Biodome (Canada). Int. J. Syst. Evol. Microbiol. 54, 269–273. https://doi.org/10.1099/ijs.0.02793-0.
Lai, Q., Qiao, N., Wu, C., Sun, F., Yuan, J., Shao, Z., 2010. Stappia indica sp. nov., isolated from deep seawater of the Indian Ocean. Int. J. Syst. Evol. Microbiol. 60, 733–736. https://doi.org/10.1099/ijs.0.013417-0.
Liu, J.J., Zhang, X.Q., Pan, J., Sun, C., Zhang, Y., Li, C.Q., Zhu, X.F., Wu, M., 2013. Aequorivita viscosa sp. nov., isolated from an intertidal zone, and emended descriptions of Aequorivita antarctica and Aequorivita capsosiphonis. Int. J. Syst. Evol. Microbiol. 63, 3192–3196. https://doi.org/10.1099/ijs.0.049635-0.
Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal; Vol 17, No 1 Next Gener. Seq. Data Anal. 17 (1), 10.
Mustakhimov, I.I., Reshetnikov, A.S., But, S.Y., Rozova, O.N., Khmelenina, V.N., Trotsenko, Y.A., 2019. Engineering of Hydroxyectoine Production based on the Methylomicrobium alcaliphilum. Appl. Biochem. Microbiol. 55, 626–630. https://doi.org/10.1134/S0003683819130015.
Pastor, J.M., Salvador, M., Argandoña, M., Bernal, V., Reina-Bueno, M., Csonka, L.N., Iborra, J.L., Vargas, C., Nieto, J.J., Cánovas, M., 2010. Ectoines in cell stress protection: Uses and biotechnological production. Biotechnol. Adv. 28, 782–801. https://doi.org/10.1016/j.biotechadv.2010.06.005.
Pérez, V., Moltó, J.L., Lebrero, R., Muñoz, R., 2021. Ectoine Production from Biogas in Waste Treatment Facilities: A Techno-Economic and Sensitivity Analysis. ACS Sustainable Chem. Eng. 9 (51), 17371–17380.
Pieja, A.J., Morse, M.C., Cal, A.J., 2017. Methane to bioproducts: the future of the bioeconomy? Curr. Opin. Chem. Biol. 41, 123–131. https://doi.org/10.1016/j.cbpa.2017.10.024.
Rocha-Rios, J., Quijano, G., Thalasso, F., Revah, S., Muñoz, R., 2011. Methane biodegradation in a two-phase partition internal loop airlift reactor with gas recirculation. J. Chem. Technol. Biotechnol. 86, 353–360. https://doi.org/10.1002/jctb.2523.
Rodero, M. del R., Carmona-Martínez, A.A., Martínez-Fraile, C., Herrero-Lobo, R., Rodríguez, E., García-Encina, P.A., Peña, M., Muñoz, R., Unpublished results. Ectoines production from biogas in pilot bubble column bioreactors and their subsequent extraction via bio-milking.
Rodríguez, Y., Firmino, P.I.M., Pérez, V., Lebrero, R., Muñoz, R., 2020. Biogas valorization via continuous polyhydroxybutyrate production by Methylocystis hirsuta in a bubble column bioreactor. Waste Manag. 113, 395–403. https://doi.org/10.1016/j.wasman.2020.06.009.
Rowe, J., Yarbrough, J., Rake, J., Eagon, R., 1979. Nitrite Inhibition of Aerobic Bacteria. Curr. Microbiol. 2, 51–54.
Sander, R., 2015. Compilation of Henry's law constants (version 4.0) for water as solvent. Atmos. Chem. Phys. 15, 4399–4981. https://doi.org/10.5194/acp-15-4399-2015.
Sauer, T., Galinski, E.A., 1998. Bacterial milking: A novel bioprocess for production of compatible solutes. Biotechnol. Bioeng. 57, 306–313. https://doi.org/10.1002/(SICI)1097-0290(19980205)57:3<306::AID-BIT7>3.0.CO;2-L.
Semrau, J.D., Dispirito, A.A., Yoon, S., 2010. Methanotrophs and copper. FEMS Microbiol. Rev. 34, 496–531. https://doi.org/10.1111/j.1574-6976.2010.00212.x.
Serejo, M.L., Posadas, E., Boncz, M.A., Blanco, S., García-Encina, P., Muñoz, R., 2015. Influence of biogas flow rate on biomass composition during the optimization of biogas upgrading in microalgal-bacterial processes. Environ. Sci. Technol. 49, 3228–3236. https://doi.org/10.1021/es5056116.
Shchukin, V.N., Khmelenina, V.N., Eshinimayev, B.T., Suzina, N.E., Trotsenko, Y.A., 2011. Primary characterization of dominant cell surface proteins of halotolerant methanotroph Methylomicrobium alcaliphilum 20Z. Microbiology 80, 608–618. https://doi.org/10.1134/S0026261711050122.
Shmareva, M.N., Doronina, N.V., Tarlachkov, S.V., Vasilenko, O.V., Trotsenko, Y.A., 2018. Methylophaga muralis Bur 1, a haloalkaliphilic methylotroph isolated from the Khilganta soda lake (Southern Transbaikalia. Buryat Republic). Microbiol. (Russian Fed. 87, 33–46. https://doi.org/10.1134/S0026261718010162.
Singh, A., Jangir, P.K., Kumari, C., Sharma, R., 2012. Genome Sequence of Nitratireductor aquibiodomus Strain RA22. J. Bacteriol. 194, 6307. https://doi.org/10.1128/JB.01510-12.
Van-Thuoc, D., Guzmán, H., Quillaguamán, J., Hatti-Kaul, R., 2010. High productivity of ectoines by Halomonas boliviensis using a combined two-step fed-batch culture and milking process. J. Biotechnol. 147, 46–51. https://doi.org/10.1016/j.jbiotec.2010.03.003.
Villeneuve, C., Martineau, C., Mauffrey, F., Villemur, R., 2013. Methylophaga nitratireducenticrescens sp. nov. and Methylophaga frappieri sp. nov., isolated from the biofilm of the methanol-fed denitrification system treating the seawater at the Montreal Biodome. Int. J. Syst. Evol. Microbiol. 63, 2216–2222. https://doi.org/10.1099/ijs.0.044545-0.
Wiesenburg, D.A., Guinasso, N.L., 1979. Equilibrium Solubilities of Methane, Carbon Monoxide, and Hydrogen in Water and Sea Water. J. Chem. Eng. Data 24 (4), 356–360.
Zhong, Z.P., Liu, Y., Wang, F., Zhou, Y.G., Liu, H.C., Liu, Z.P., 2016. Lacimicrobium alkaliphilum gen. nov., nov., sp. nov., a member of the family Alteromonadaceae isolated from a salt lake. Int. J. Syst. Evol. Microbiol. 66, 422–429. https://doi.org/10.1099/ijsem.0.000735.