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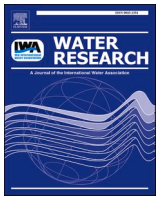

Ectoines production from biogas in pilot bubble column bioreactors and their subsequent extraction via bio-milking

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ABSTRACT

Despite the potential of biogas from waste/wastewater treatment as a renewable energy source, the presence of pollutants and the rapid decrease in the levelized cost of solar and wind power constrain the use of biogas for energy generation. Biogas conversion into ectoine, one of the most valuable bioproducts (1000 €/kg), constitutes a new strategy to promote a competitive biogas market. The potential for a stand-alone 20 L bubble column bioreactor operating at 6% NaCl and two 10 L interconnected bioreactors (at 0 and 6% NaCl, respectively) for ectoine production from biogas was comparatively assessed. The stand-alone reactor supported the best process performance due to its highest robustness and efficiency for ectoine accumulation (20–52 $mg_{ectoine}/g_{VSS}$) and CH_4 degradation (up to 84%). The increase in N availability and internal gas recirculation did not enhance ectoine synthesis. However, a 2-fold increase in the internal gas recirculation resulted in an approximately 1.3-fold increase in CH_4 removal efficiency. Finally, the recovery of ectoine through bacterial bio-milking resulted in efficiencies of >70% without any negative impact of methanotrophic cell recycling to the bioreactors on CH_4 biodegradation or ectoine synthesis.

1. Introduction

In recent years, the increased generation of municipal solid waste and wastewaters has resulted in the construction of new anaerobic digestion plants (up to 18,113 plants in Europe in 2019). Consequently, there is a higher availability of biogas in the global energy market (European Biogas Association, 2020). Biogas has been traditionally considered a green bioenergy source due to its high methane content (40–75%) and is directly used for electricity and/or heat generation in internal combustion engines or turbines (Ryckebosch et al., 2011). Nevertheless, the high investment costs of gas engines/turbines (400–1100 €/kW) and the rapid decrease in solar and wind energy prices (82% and 39% decrease from 2010 to 2019, respectively), jeopardize the widespread use of biogas as an energy vector (Pérez et al., 2020). As a result, biogas is eventually being flared or vented into the atmosphere in small-medium scale anaerobic digestion facilities, thus increasing the greenhouse gas emissions in the waste management sector. Therefore, the use of biogas as a feedstock for CH_4 bioconversion into high added value products, such as single-cell protein, methanol, biopolymers, and ectoine, represents an new opportunity to upgrade the value of the main by-product from anaerobic digestion while preventing the uncontrolled release of CH_4 into the atmosphere (Kapoor et al., 2020).

Currently, ectoine (1,4,5,6,tetra-2-methyl-4-pyrimidinecarboxylic acid) is one of the most profitable products produced by microorganisms, with a market sale value of approximately 600–1000 €/kg (Becker and Wittmann, 2020; Pérez et al., 2021). A recent techno-economic analysis revealed that the production costs of ectoine from biogas could range from 158 to 275 €/kg, with negligible changes with variations in energy and water prices, while the total investment costs could amount to 4.21 M€ (Pérez et al., 2021). The large difference between the sale values and the production costs makes this process highly profitable, with a payback time of less than 3 years. Ectoine is produced in

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extremophile microorganisms to protect them under harsh environments, such as hyper-osmotic stress or extreme temperatures, both high and low (Czech et al., 2018; Pastor et al., 2010). Ectoine has the property of binding to water and creating a complex with a protective effect on proteins and cell membranes against these severe environmental conditions (Becker and Wittmann, 2020). Its hydroxylated derivative, hydroxyectoine – which is often synthesized in combination with ectoine by extremophilic microorganisms – also acts as a protector against desiccation and heat stress, with an even greater capacity than ectoine (Liu et al., 2021). For this reason, these extremolites are widely applied in the cosmetic industry, mainly as an ingredient in skin creams and lotions, and in the pharmaceutical industry as a component in nasal sprays, eye drops, and lung inhalation fluids, among others (Becker and Wittmann, 2020). Their conventional production at industrial scale is based on the ability of the *γ-Proteobacterium Halomonas elongata* to synthetize ectoine and hydroxyectoine in a high salinity medium and which releases these extremolites when exposed to an osmotic downshock in a process called "bacterial milking" or "bio-milking" (Pastor et al., 2010; Sauer and Galinski, 1998). Under conditions of high osmotic stress, these microorganisms accumulate ectoines either by biosynthesis or importing them from the environment to reach an osmotic strength in the cytoplasm close to that of the surrounding medium, thus preventing water loss from the cell (Grammann et al., 2002). When the external salinity decreases, the cell counteracts the excessive water entrance by excreting the previously accumulated ectoines through a temporary opening of mechanosensitive channels which prevents cell breakdown by decreasing the turgor magnitude (Czech et al., 2022). Today, the "leaking mutant" of *H. elongata* is applied since it enhances ectoine production and allows for the excretion of ectoine from the cell to the medium without the osmotic downshock step by disabling the ectoine degradation pathway and removing the TRAP transporter TeaABC for ectoine uptake (Hobmeier et al., 2022; Kunte et al., 2014). Despite its potential, this process exhibits high operation costs as a result of the need of a high quality carbon feedstock (i.e. glucose).

Previous studies have identified the genes of the ectoine biosynthesis pathway in halotolerant methanotrophs such as *Methylobacter marinus* 7C, *Methylomicrobium kenyense* AMO1^T, and *Methylomicrobium alcaliphilum* 20Z (Czech et al., 2018; Reshetnikov et al., 2011). Nevertheless, the ectoine productivities achieved were low compared to those reported in the commercial industrial process (Pastor et al., 2010) which was mainly attributed to the poor aqueous solubility of methane, limiting the process operation at high cell densities (Cantera et al., 2020). In this context, multiple bioreactor configurations for CH_4 abatement have been tested, with bubble column reactors supporting both efficient CH_4 mass transfer rates and the suspended growth of biomass needed for ectoine downstream processing (Cantera et al., 2018, 2017a). Bubble column bioreactors can be designed with internal gas recirculation in order to enhance methane gas-liquid mass transfer and, consequently, ectoine and hydroxyectoine productivities (Rodríguez et al., 2020). The results obtained by Cantera et al. (2017b) suggested that process operation based on a stand-alone reactor at high salinity with *M. alcaliphilum* 20Z reduces the content of intracellular extremolites as a result of culture adaptation, and the use of 2-stage cultivation (operating sequentially at a low and high salt content) could eventually overcome this limitation. In addition, Cantera et al. (2020) observed an inhibition of CH_4 biodegradation during ectoine production as a result of the accumulation of toxic metabolites. This highlighted the need to operate at high dilutions rates and design strategies to retain the slow-growth halotolerant methanotrophs in the bioreactor. This slow growth of methanotrophic bacteria also entails a challenge during ectoine bio-milking, since the feasibility of bacterial recycling to the bioreactor after ectoine extraction has not yet been assessed.

This study aims to systematically compare the performance of a stand-alone 20 L high mass transfer membrane bioreactor operating at 6% NaCl and two 10 L membrane bioreactors interconnected in series (at 0% and 6% NaCl, respectively) during biogas bioconversion into ectoine. Here, the influence of the nitrogen loading rate, dilution rate, and gas internal recirculation on CH_4 bioconversion into ectoines was evaluated. Finally, an innovative bio-milking process involving ectoine extraction and methanotrophic cell recycling was validated.

2. Materials and methods

2.1. Mineral salt medium

The mineral salt medium (MSM) was composed of (per L of solution): 100 mL of NMS2 (10 ×) solution, 20 mL of phosphate buffer, 45 mL of $NaHCO_3$ buffer (84 g/L), 26 μL of $Na_2WO_2{\cdot}2H_2O$ solution (2.7 g/L), 2 mL of trace elements solution, and the corresponding amount of NaCl (based on the mineral salt medium for haloalkaliphilic methane-oxidizing bacteria proposed by Kalyuzhnaya et al. (2008)). The NMS2 (10 ×) solution contained (per L): 2.0 g $MgSO_4{\cdot}7H_2O$, 0.13 g $CaCl_2{\cdot}2H_2O$, and the corresponding amount of KNO_3, according to the N concentration established during the operational stages. The phosphate buffer solution contained (per L): 5.44 g KH_2PO_4 and 6.25 g $Na_2HPO_4{\cdot}2H_2O$. The trace elements solution contained (per L): 5.0 g Na_2EDTA, 0.076 g $CuCl_2{\cdot}2H_2O$, 2.0 g $FeSO_4{\cdot}7H_2O$, 0.1 g $ZnSO_4{\cdot}7H_2O$, 0.02 g $NiCl_2{\cdot}6H_2O$, 0.2 g $CoCl_2{\cdot}6H_2O$, 0.035 g $Na_2MoO_4{\cdot}2H_2O$, 0.03 g $MnCl_2{\cdot}4H_2O$, and 0.03 g H_3BO_3. All reagents were obtained from Panreac Applichem (Spain) and COFARCAS (Spain) with a purity >99%.

2.2. Experimental set-up

Two interconnected PVC rectangular bubble column bioreactors (height: 67 cm, length: 20 cm, width: 10 cm) with 10 L of working volume operating at an NaCl content of 0% and 6% (henceforth referred to as R1 and R2, respectively), and a 20 L stand-alone PVC rectangular bubble column bioreactor (height: 83 cm, length: 20 cm, width: 20 cm) operated at an NaCl concentration of 6% (named R3) were used to systematically assess the best bioreactor configuration in terms of ectoine production and methane degradation. A custom-made plastic diffuser with a rubber membrane (0.5 mm pore size) was installed at the bottom of the bioreactors to allow gas sparging. The inlet gas was composed of a mixture of H_2S-free synthetic biogas (70% CH_4 and 30% CO_2, Carburos Metalicos S.A. Spain) supplied via a mass flow controller (Aalborg, USA) and air to mimic a diluted biogas stream with a CH_4 content of 5% (below the explosion limit). The two interconnected bioreactors were fed with the diluted biogas stream at a flowrate of ≈0.21 L/min, while R3 was operated with a gas flowrate of ≈0.38 L/min, which resulted in empty bed residence times of 47 (R1 and R2) and 52 (R3) min. Moreover, an internal recirculation of the outlet gas was implemented to improve CH_4 mass transfer in the bioreactors (Rocha-Rios et al., 2011). The bioreactors were interconnected to a water trap and a jacketed water condenser operated at 10 °C prior to the H5P3P-1 gas recirculation compressor (ElectroAD, Spain) in order to prevent water condensation and salt/biomass accumulation in the gas line (Fig. 1). A fixed volume of biomass-free medium was replaced with fresh MSM daily in order to avoid microbial inhibition by metabolite accumulation and to prevent nutrient limitation. A membrane module (Koch Membrane Systems, Germany) with a pore size of 0.03 μm was located inside the bioreactors to retain the microbial cells during medium replacement.

2.3. Operational procedures

Three operational strategies were implemented in order to guarantee the availability of the nitrogen and carbon sources for ectoine synthesis by methanotrophs (Table 1). The three bioreactors were initially inoculated with a methanotrophic bacterial consortium acclimated to 6% NaCl MSM enriched from a salt lagoon (Burgos, Spain) (Carmona-Martínez et al., 2021). In this study, a methanotrophic consortium was

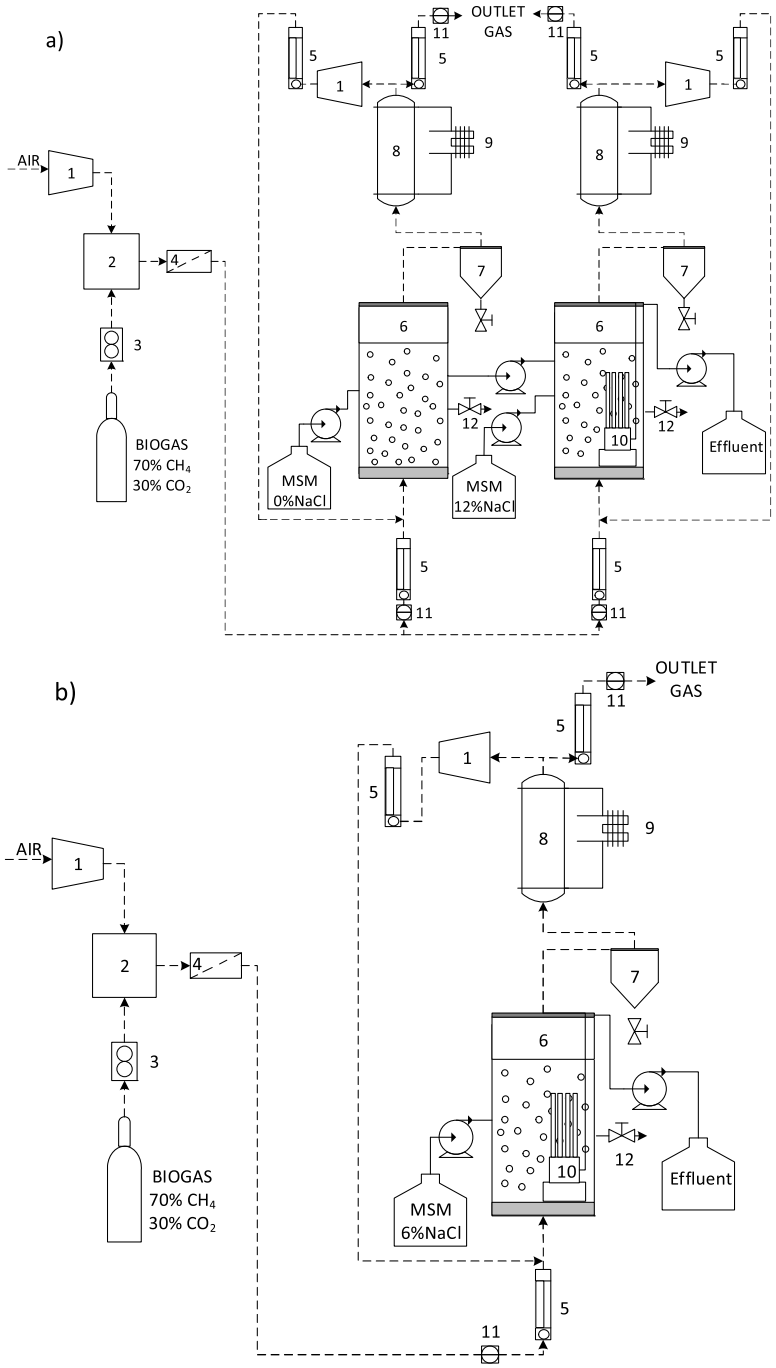

Fig. 1. Schematic diagram of the experimental set-up: a) two bioreactors interconnected in series, b) stand-alone bioreactor. (1) Compressor, (2) Mixing chamber, (3) Mass flow controller, (4) Gas filter, (5) Rotameter, (6) Bubble column bioreactor, (7) Water trap, (8) Condenser, (9) Thermostatic bath, (10) Membrane module, (11) Gas sampling port, (12) Liquid sampling port.

chosen over a pure culture because it is more economically viable on an industrial scale as it avoids the use of sterile conditions and has previously been shown to produce more ectoine than the pure culture *M. alcaliphilum* 20Z (Cantera et al., 2020). Unfortunately, biological replicates were not performed in this study. In Stage I, 1 L/d of filtered cultivation broth from the two interconnected bioreactors (withdrawn from R2) and R3 was replaced with fresh MSM with a N concentration of ~140 mg/L, operating at an internal gas recirculation flowrate of 3.2 (R1 and R2) and 5.8 (R3) L/min (~15 times the inlet gas flowrate). In this context, 0.5 L of the whole cultivation broth of R1 and 0.5 L of fresh MSM at 12% NaCl were pumped daily into R2 in order to maintain the volume and NaCl concentration (6%) in R2. During Stage II, the N concentration in the MSM was tripled and the MSM replaced was increased to 2 L per day to assure enough availability of N for biomass growth and ectoine synthesis. In Stage III, the internal gas recirculation in the bioreactors was doubled (6.3 L/min for R1 and R2, and 11.6 L/min for R3). Stage III was carried out without implementing the bio-milking process (IIIa) and implementing the bio-milking process in R2 and R3 (IIIb), as described in Section 2.4.

Liquid samples of 200 mL from the cultivation broth of the bioreactors were withdrawn three times per week to monitor volatile suspended solids (VSS) concentration, pH, dissolved TN and TOC, and intra-cellular ectoine and hydroxyectoine concentration. Gas samples of 100 μL from the inlet and the outlet of the bioreactors were also drawn

Table 1
Operational conditions applied during the three operational stages.

Operational stage	Reactor	Feed (L/d)	Internal gas recirculation (L/min)	N-NO_3 MSM (mg/L)
I	R1	0.5 MSM 0% NaCl	3.2	140
	R2	0.5 MSM 12% NaCl + 0.5 cultivation broth R1	3.2	140
	R3	1 MSM 6% NaCl	5.8	140
II	R1	1 MSM 0% NaCl	3.2	420
	R2	1 MSM 12% NaCl + 1 cultivation broth R1	3.2	420
	R3	2 MSM 6% NaCl	5.8	420
III	R1	1 MSM 0% NaCl	6.3	420
	R2	1 MSM 12% NaCl + 1 cultivation broth R1	6.3	420
	R3	2 MSM 6% NaCl	11.6	420

three times per week to determine gas concentrations by GC-TCD using gas tight syringes. The gas and liquid analyses were carried out in duplicate. Average values and standard deviations were calculated based on these duplicate technical measurements. Despite the fact that no biological replicates were performed in this work, the duration of the operational strategies was long enough to ensure steady state and thus, the reproducibility of the data. At this point, it should also be stressed that the performance of the systems was mostly governed by gas-liquid mass transfer and therefore, the influence of the microbiology was limited.

2.4. Ectoine and hydroxyectoine extraction via bio-milking

The time of incubation under hyposmotic conditions and the ratio *water:cultivation broth* needed for the cells to release the intracellular ectoine and hydroxyectoine was optimized. For this purpose, 40 mL of cultivation broth from R3 were initially centrifuged (5 min, 10,000 rpm) in 50 mL falcon tubes. The supernatant was discharged and different volumes of distilled water were added to the biomass pellet (10, 15, and 20 mL) to re-suspend the cultures, which were maintained under gentle shaking for 30 min. Samples of the re-suspended cultivation broth were periodically taken and immediately filtered through 0.22 μm pore size filters prior ectoine and hydroxyectoine analysis. The assays were carried out in duplicate.

Following bio-milking optimization, 3 L of the cultivation broth of R2 and R3 were daily withdrawn during Stage IIIb and centrifuged at 10,000 rpm for 5 min (Sorvall, United States). After the centrifugation of the cultivation broth, a volume of distilled water was added to the pellet with a ratio of 1.5/4 (volume distilled water/volume cultivation broth) and mixed for 5 min. Then, a second centrifugation step at 10,000 rpm for 5 min was carried out in order to separate the biomass from the ectoine-containing supernatant. Finally, this biomass pellet and the salt medium from the first centrifugation step were returned to the bioreactors, and a sample of the supernatant from the second centrifugation step was filtered through a 0.22 μm filter for extracellular ectoine and hydroxyectoine analysis after the downshock.

2.5. Analytical procedures

CH_4 gas concentrations were quantified using a Bruker 430 GC-TCD (Palo Alto, USA) equipped with CP-Molsieve 5A (15 m × 0.53 mm × 15 mm) and CP-PoraBOND Q (25 m × 0.53 mm × 10 mm) columns with helium (13 mL/min) as the carrier gas. The pressure in the inlet stream was monitored with a differential pressure sensor (Ifm Electronic (Essen, Germany)). The VSS concentration was determined according to standard methods (Eaton et al., 2005). The pH was analyzed using a Hach Sension+ PH3 pHmeter (Düsseldorf, Germany). Dissolved TOC and TN concentrations were quantified following sample filtration through a 0.45 μm pore size filter using a TOC-VCSH analyser (Shimadzu, Japan) interconnected to a TNM-1 detector.

Extracellular ectoine and hydroxyectoine analysis were only performed after the hipo-osmotic shock in Stage IIIb, while the intracellular content of ectoine and hydroxyectoine of the biomass was reported during all stages since it was previously demonstrated that under a concentration of 6% NaCl the extracellular content of ectoine was insignificant (Cantera et al., 2017a). The intracellular ectoine and hydroxyectoine concentration was analysed using 2 mL of cultivation broth centrifuged at 9000 × g for 10 min in an Eppendorf tube. After supernatant discharge, the pellet was washed twice with a solution of 6% NaCl in milli-Q water prior to the addition of 1.8 mL of ethanol at 70% and 25 ± 5 mg of 0.1-mm-diameter zirconia/silica beads (BioSpec, Spain). The bacterial cells were disrupted using a Mini-BeadBeater-16 (BioSpec, Spain) at 1048 × g for 10 min. The final suspension was centrifuged at 9000 × g for 15 min and filtered through 0.22 μm filters for ectoine and hydroxyectoine analysis. Intracellular and extracellular ectoine and hydroxyectoine were measured by HPLC-UV according to Cantera et al. (2020). The quantification of both components was performed using standards of commercially available ectoine ((*S*)-b-2-methyl-1,4,5,6-tetrahydro pyrimidine-4-carboxylic acid, purity ≥ 95%, Sigma Aldrich, USA) and hydroxyectoine ((4S,5S)−5-Hydroxy-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid, purity ≥ 95%, Sigma Aldrich, USA) dissolved in ethanol at 70%. The specific concentrations ($mg_{ectoine}/g_{biomass}$, $mg_{hydroxyectoine}/g_{biomass}$) were estimated using the corresponding VSS concentration (g/L) of the cultivation broth.

2.6. Bacterial community analysis

Samples from the bacterial inoculum and the cultivation broth present in the three bioreactors at the end of each operational stage were withdrawn for the determination of the microbial population structure and composition by means of 16S rDNA gene sequencing. The centrifuged biomass was used for DNA extraction using the MagNA Pure LC DNA Isolation Kit III (Roche Molecular Systems, Inc, Switzerland) according to the manufacturer's instructions. Sequencing of the extracted DNA was performed using a 2 × 300pb paired-end run on an Illumina Miseq Sequencer at FISABIO (Valencia, Spain). Prior to sequencing, 16S rDNA gene amplicons were obtained following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15,044,223 Rev. A Illumina, Inc., San Diego, CA, USA). For this purpose, genomic DNA (5 ng μL^{-1}) was used to amplify the V3-V4 region of the 16S rDNA gene via PCR using the primers from Klindworth et al. (2013). Illumina adapter overhang nucleotide sequences were attached to the gene-specific sequences. After 16S rDNA gene amplification, the mutiplexing step was carried out using Nextera XT Index Kit (FC-131–1096). 1 μl of the PCR product was run on a Bioanalyzer DNA 1000 chip for size verification of the libraries (expected size ~550 bp). After size verification, the libraries were sequenced.

The quality of the sequences obtained was assessed using PRINSEQ program (Schmieder and Edwards, 2011). The resulting sequence read files were analysed using the QIIME2 platform (Caporaso et al., 2010) in terms of taxonomic assignments; denoising, paired-ends joining, and chimera filtering were done using the DADA2 pipeline (Callahan et al., 2016). Taxonomic affiliations were assigned using the Naive Bayesian classifier integrated in QIIME2 plugins; SILVA138 was the reference database (Quast et al., 2013) and a 97% similarity cut-off was used for cluster reads for taxonomic classification. Additional data was obtained using an ad hoc pipeline written in the R Statistics environment, making use of several open-source libraries (GData (Warnes et al., 2013) and Vegan (Oksanen et al., 2011)).

3. Results and discussion

3.1. Methane biodegradation

The CH_4 elimination capacity (CH_4-EC) and CH_4 removal efficiency (CH_4-RE) in R1 were unstable during Stage I, fluctuating between 5.0–10.9 g CH_4 /(m^3 h) and 15–36%, respectively, likely due to the low biomass concentrations (Fig. 2). In Stage II, both parameters increased to average values of 15.6 ± 2.3 g CH_4 /(m^3 h) and 50±5%, likely due to the increase in N supply and the higher biomass concentrations compared to Stage I. In Stage III, the increase of the internal gas recirculation from 3.2 to 6.3 L/min improved CH_4 elimination to stable values of 23.2 ± 1.4 g CH_4 /(m^3 h) and 74±3% for CH_4-EC and CH_4-RE, respectively. The higher turbulence in the cultivation broth due to the increase in the gas recirculation entailed higher volumetric mass transfer coefficients, and consequently an increase in CH_4 gas-liquid mass transfer (Kraakman et al., 2011). Similarly, Rodríguez et al. (2020) reported an increase in CH_4-EC and CH_4-RE by a factor of 1.3 and 1.2, respectively, by increasing the internal gas recirculation from 15 to 30 times the inlet flow rate.

CH_4-ECs and CH_4-REs of 10.2–16.7 g CH_4 /(m^3 h) and 30–50%, respectively, were recorded in R2 during Stage I (Fig. 2). Unexpectedly, when the N source in the feed and the volume of R1 pumped to R2 was increased in Stage II, CH_4-ECs and CH_4-REs decreased down to 5.3 g CH_4 /(m^3 h) and 18%, respectively. This low CH_4 degradation could be attributed to the accumulation of inhibitory compounds such as nitrite, methanol, or formate, which was supported by the high TOC concentration recorded during this stage (204±23 mg TOC/L) in the cultivation broth (Cantera et al., 2020). On the other hand, CH_4-ECs and CH_4-REs increased up to 18.3 g CH_4 /(m^3 h) and 54%, respectively, at the beginning of Stage III as a result of the increase in the internal gas recirculation. However, CH_4-EC and CH_4-RE gradually decreased down to 10.1 g CH_4 /(m^3 h) and 35%, values lower than those recorded in R1 during Stages II and III. The CH_4-EC values obtained in R2 at 6% NaCl were in the range of those reported by Cantera et al. (2017a) (5–22.4 g CH_4 /(m^3 h)) in stirred tank reactors treating diluted methane emissions with an NaCl concentration of 6%.

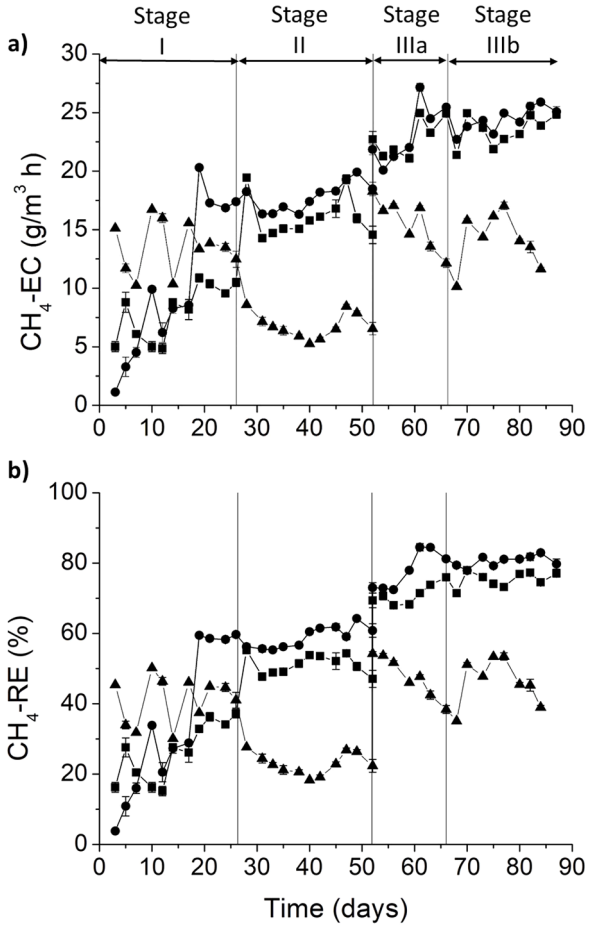

Fig. 2. Time course of (a) CH_4 Elimination Capacity and (b) CH_4 Removal Efficiency in R1 (■), R2 (▲) and R3 (●). The error bars represent the standard deviation between duplicate technical measurements.

R3 showed a steady increase in CH_4-EC and CH_4-RE throughout Stage I, from less than 1.2 g CH_4 /(m^3 h) and 4% to a stable CH_4 consumption of 18.2 ± 0.5 g CH_4 /(m^3 h) and 59±2% of CH_4-EC and CH_4-RE, respectively (Fig. 2). Similar CH_4-ECs and CH_4-REs were observed during Stage II at increasing N loading rates. Unfortunately, these CH_4-ECs were lower than those achieved in previous studies under similar internal gas recirculation ratios, mainly due to the lower gas residence times. For instance, Rodríguez et al. (2020) reported CH_4-ECs of 58 g CH_4 /(m^3 h) at a Q_R/Q_{INLET}=15 in a 2.5 L bubble column bioreactor operated with *Methylocystis hirsuta* at a gas residence time of 30 min and with 2 μm metallic gas diffusers. On the other hand, the increase in the internal gas recirculation in Stage III mediated an increase in CH_4-EC and CH_4-RE up to constant values of 23.9 ± 1.9 g CH_4 /(m^3 h) and 79 ±4%, respectively.

Overall, no negative effect on the CH_4 consumption was observed when the bio-milking process was implemented in the bioreactors in Stage IIIb, with R3 being the bioreactor that supported the highest CH_4 mass transfer performance (Fig. 2b). The stable values in terms of CH_4 removal achieved under steady state in the operational stages demonstrated the consistency of the values hereby obtained.

3.2. Ectoine and hydroxyectoine production

Despite the fact that bioreactor R1 supported biomass growth, the methanotrophic consortium barely accumulated ectoine or hydroxyectoine since cells were not subjected to salinity stress (0% NaCl, Fig. 3). On the other hand, the bacterial consortium in R2 and R3 showed ectoine accumulation as a response to the saline environment (6% NaCl). Interestingly, ectoine concentration in the cells gradually decreased over time during Stage I (Fig. 3a). Indeed, the highest values of specific ectoine concentrations were recorded in the first two weeks of operation of R2 and R3 (87 ± 0 and 52 ± 3 $mg_{ectoine}/g_{VSS}$, respectively). This phenomenon was initially attributed to the gradual biomass growth, which could have induced a limitation in nitrogen or carbon, key substrates for the biosynthesis of hydroxyectoine and ectoine. However, the additional supplementation of nitrogen in Stage II and the increase in carbon availability via the increase in the internal gas recirculation in Stage III did not contribute to an increase in ectoine content in the cultivation broth, which remained almost constant at 13 ±4 and 27±4 $mg_{ectoine}/g_{VSS}$ in R2 and R3, respectively, during Stages II and III. Similarly, Cantera et al. (2017a) observed a peak in the intracellular ectoine content of *M. alcaliphilum* 20Z during the first week of operation, followed by a sharp decrease in ectoine accumulation. This event was associated with an initial overexpression of the ectABC operon due to the osmotic shock prior to ectoine assimilation by the bacterial metabolism in the following days. In our particular study, the hyperosmotic shock experienced by the biomass transferred from R1 to R2, and the biomass recirculated after bio-milking (Stage IIIb) did not result in an enhanced synthesis of ectoine. Overall, the ectoine yields herein obtained were slightly lower than those achieved by Carmona et al. (2021), using a similar methanotrophic enriched consortium under similar NaCl content and temperature (30 $mg_{ectoine}/g_{VSS}$). These differences could be attributed to the higher CH_4 concentrations used by Carmona et al. (2021) (9% vs 5% CH_4 in this study), which typically increase the synthesis of ectoine (Cantera et al., 2016).

The amount of hydroxyectoine synthetized in R2 and R3 was lower than that of ectoine during the three operational stages, since

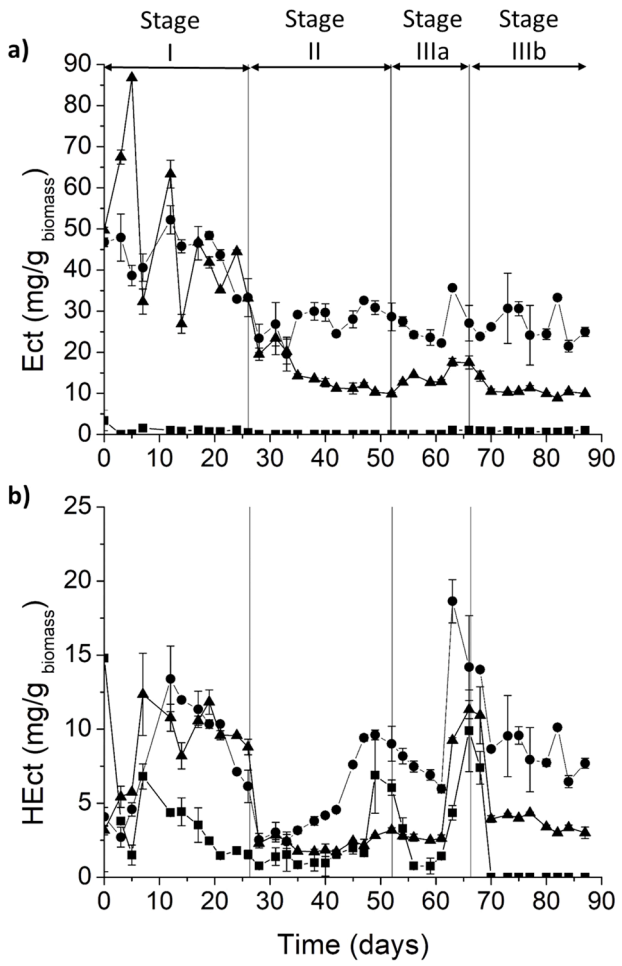

Fig. 3. Time course of intracellular (a) ectoine (Ect) and (b) hydroxyectoine (HEct) yields in the cultivation broth of R1 (■), R2 (▲) and R3 (●). The error bars represent the standard deviation between duplicate technical measurements.

hydroxyectoine production requires an additional step for ectoine conversion via a stereo-specific hydroxylation catalyzed by EctD (Bursy et al., 2007). During Stage I, the maximum hydroxyectoine concentration in the cultivation broth of R2 and R3 was 12±3 and 13±2 $mg_{hydroxyectoine}/g_{VSS}$, respectively. These maximum concentrations were observed during the first days of operation (Fig. 3). During Stage II, the hydroxyectoine concentration in the cultivation broth of R2 remained constant at 2 ± 1 $mg_{hydroxyectoine}/g_{VSS}$. On the other hand, the increase in the N supply in Stage II entailed a gradual increase in hydroxyectoine concentration up to 10±1 $mg_{hydroxyectoine}/g_{VSS}$ in R3. During Stage IIIa, the increase in the internal gas recirculation resulted in maximum hydroxyectoine concentrations of 12±2 and 18±1 $mg_{hydroxyectoine}/g_{VSS}$ in R2 and R3, respectively. Finally, constant hydroxyectoine concentrations of 4 ± 1 and 8 ± 1 $mg_{hydroxyectoine}/g_{VSS}$ were recorded in R2 and R3, respectively, as a result of cell bio-milking in Stage IIIb (Fig. 3b).

The hydroxyectoine contents obtained herein were in the range of those previously reported by Cantera et al. (2018) using an enrichment of haloalkaliphilic methanotrophs at 6% NaCl (3.8–13.3 $mg_{hydroxyectoine}/g_{VSS}$). Nevertheless, the maximum hydroxyectoine concentrations and hydroxyectoine/ectoine ratios (43:57) recorded in this study were lower than those reported in engineered *M. alcaliphilum* 20Z (22 $mg_{hydroxyectoine}/g_{wet\_cells}$ and hydroxyectoine/ectoine ratio of 76:24) and *Hansenula polymorpha* (58 $mg_{hydroxyectoine}/g_{VSS}$ and ~100% conversion of ectoine to hydroxyectoine) (Eilert et al., 2013; Mustakhimov et al., 2019).

3.3. Bioreactor performance

The pH of the cultivation broth in the three bioreactors remained constant at 8.4 ± 0.2, 8.0 ± 0.1, and 8.0 ± 0.2 for R1, R2, and R3, respectively. Despite the continuous addition of CO_2 (acidic gas) from biogas, the pH in the cultivation broth of the bioreactors remained slightly higher than that of MSM (7.9 ± 0.2), which could be attributed to the synthesis of basic extracellular metabolites (De Carvalho and Fernandes, 2010). These values of pH were in the range of those previously reported in the literature as optimal for methane-oxidizing bacteria (4–10) (Reddy et al., 2020).

The biomass concentration (measured as VSS) in R1 (0% NaCl) remained constant under steady state during each operational stage since part of the biomass was pumped daily from R1 to R2 (0.5 L/d during Stage I and 1 L/d during Stages II, IIIa, and IIIb). In this context, the increase in the N concentration in the MSM entailed an increase in biomass concentration in R1 from 0.5 to 1.0 g VSS/L (Fig. 4a). Moreover, the increase in the internal gas recirculation during Stage III also involved an increase in the biomass concentration in R1 up to 2 g/L as a result of the higher CH_4 mass transfer in the bioreactor. On the other hand, R2 and R3 accumulated biomass as a result of the effective biomass retention mediated by the membrane module. Interestingly, R3 supported similar concentrations of biomass when compared to R2 during all the operational stages, in spite of the net biomass input from R1 to R2. In this context, R2 and R3 reached biomass concentrations of 1.43±0.04 and 1.37±0.07 g VSS/L, respectively, at the end of Stage I. The increase in the N concentration in the MSM and dilution rate in Stage II increased the biomass concentration up to 3 g VSS/L in R2 and R3. The plateau reached in the biomass concentration between days 40 and 52 under constant biogas supply, no nitrogen limitation, and total biomass retention suggest that the CH_4 transfer capacity of the bioreactors was not sufficient to support new biomass growth and all methane transferred was devoted to cell maintenance. The increase in CH_4 mass transfer caused by the higher internal gas recirculation induced intense biomass growth up to maximum biomass concentrations of 5.70±0.71 and 6.60±0.42 g VSS/L in R2 and R3, respectively, by the end of Stage IIIa. This suggests the occurrence of carbon limitation during Stage II. During Stage IIIb, biomass bio-milking was conducted at a constant VSS concentration of ≈ 4 g/L. In this context, the bio-milking process exerted a negative impact on biomass growth during the first days as a consequence of the severe hyperosmotic shock when cells were returned to the bioreactors. Indeed, cells entered R2 and R3 with a low ectoine content after extraction and likely burst, as suggested by the intense foaming observed in the cultivation broths. The deterioration in the concentration of biomass was more severe in R3 since the methanotrophic bacteria in R2 were inherently adapted to hyperosmotic shocks (Fig. 4a). The maximum biomass productivities achieved during Stage IIIa were 5.6 ± 2.5 and 10.4 ± 6.3 g VSS/ (m^3 h) for the two interconnected bioreactors (R1+R2) and the stand-alone bioreactor (R3), respectively. These biomass productivities were slightly higher than those reported for a mixed bacterial alkaliphilic consortium in a 2 L bubble column bioreactor (1.6–3.9 g VSS/ (m^3 h)) (Cantera et al., 2020). Unfortunately, the biomass concentrations were below the cell densities reached in the conventional industrial process for ectoine production with *H. elongata* (48 g/L) (Pastor et al., 2010).

The TOC concentration in R1 remained lower than 131±2 mg/L during the three operational stages (Fig. 4b). The TOC concentration initially increased up to 124±7 mg/L by the end of Stage I, which was attributed to bacterial acclimation, and decreased throughout Stage II, mediated by the increase in the MSM dilution rate. The increase in the internal gas recirculation in Stage III promoted higher turbulence in the cultivation broth of the bioreactor, which likely caused cell disruption and the release of cellular metabolites, thus increasing the TOC concentration. Similarly, Cantera et al. (2017a) reported shear stress on *M. alcaliphilum* 20Z at high agitation rates. Nevertheless, the TOC gradually decreased by the end of Stage III as a result of biomass

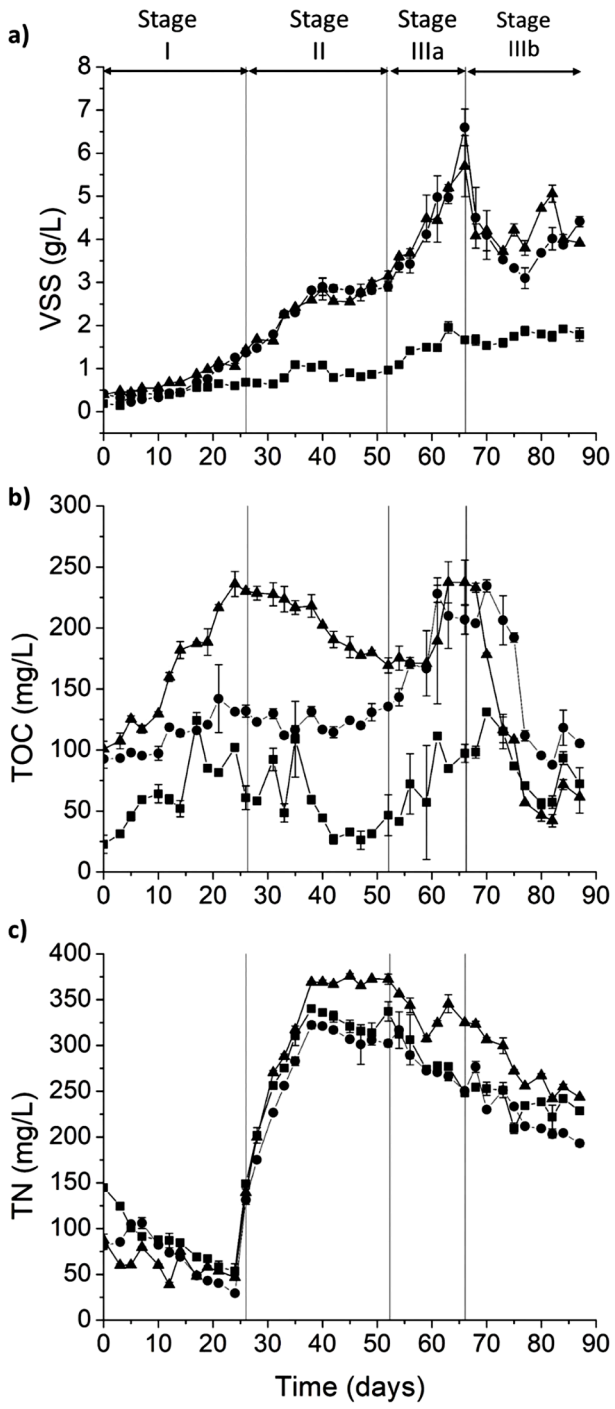

Fig. 4. Time course of the concentration of (a) volatile suspended solids (VSS), (b) total organic carbon (TOC) and (c) total nitrogen (TN) in the cultivation broth of R1 (■), R2 (▲) and R3 (●). The error bars represent the standard deviation between duplicate technical measurements.

acclimation to the prevailing operational conditions. On the other hand, the TOC concentrations in R2 were higher (up to 238±17 mg/L) than in R1, likely due to cellular lysis induced by the hyperosmotic shock when the biomass was transferred from R1 to R2. This high TOC concentration in R2 throughout most of the experiment could have exerted a negative impact on methanotrophic activity, since a TOC concentration threshold of 100 mg/L has been identified as partially inhibitory (Estrada et al., 2014; Mancebo et al., 2014). The TOC concentrations in R3 were lower than those in R2 in Stages I and II (maximum concentration of 136 mg/L) likely due to the absence of a significant biomass lysis, but increased up to similar values as those in R2 with the increase in the internal gas recirculation during Stage IIIa (Fig. 4b).

Similar TN concentrations were recorded in the three bioreactors throughout the experimental period. The initial N concentration in the MSM was not sufficient to support the active biomass growth during Stage I at the dilution rate imposed, which resulted in a gradual decrease in the nitrogen available in the cultivation broth down to 53.6 ± 8.1, 46.7 ± 1.7, and 29.4 ± 1.6 mg/L in R1, R2, and R3, respectively (Fig. 4c). In Stage II, the N concentration in the MSM and the volume of medium replaced daily were increased to avoid nitrogen limitation, thereby increasing TN concentrations in the cultivation broth to steady state values of 327.9 ± 10.9, 370.3 ± 3.7, and 311.0 ± 9.0 mg/L in R1, R2, and R3, respectively. In this regard, Rodríguez et al. (2020) observed a negative impact on methanotrophic activity with an increase in the N concentration from 276 to 552 mg N—NO_3/L due to nitrite accumulation under operation at low O_2:CH_4 ratios of 1.3:1–1.7:1. In our particular study, no pernicious effect on bacterial activity (except in R2) was observed with the increase in the TN concentration, likely due to the high O_2:CH_4 ratio set in this study (4:1) which prevented nitrite formation. Finally, the intense biomass growth observed in Stage III as a result of the enhanced CH_4 supply entailed a gradual decrease in the TN concentration in the three bioreactors (Fig. 4c).

3.4. Ectoine and hydroxyectoine bio-milking

Ectoine and hydroxyectoine were excreted very quickly and only 5 min of exposure of the cells to a salt-free medium was needed to reach the maximum concentration of ectoine and hydroxyectoine in batch assays (Fig. 5). The recovery of ectoine and hydroxyectoine ($mg_{intracellular}/mg_{released} \times 100$) was lower at water:cultivation medium ratios of 1:4 (average values of 76±2% and 48±0% for ectoine and hydroxyectoine, respectively) compared to those obtained at 1.5:4 and 2:4 (ectoine recoveries of 83±1% and 85±2%, respectively, and hydroxyectoine recoveries of 52±1% in both cases). Although similar results were obtained at ratios of 1.5:4 and 2:4, the former was preferred since it required less water consumption and ectoine and hydroxyectoine were more concentrated (224±11 $mg_{ectoine}$/L and 33±2 $mg_{hydroxyectoine}$/L at a ratio of 1.5:4 *vs* 171±7 $mg_{ectoine}$/L and 25±1 $mg_{hydroxyectoine}$/L at a ratio of 2:4), thus lowering the costs of downstream process (Fig. 5). Therefore, 5 min of exposure to a salt-free medium and a distilled water/cultivation broth ratio of 1.5/4 were selected as bio-milking operating parameters in the bioreactors during Stage IIIb.

Cell bio-milking during Stage IIIb resulted in average ectoine recoveries of 79±17% and 92±7% in R2 and R3, respectively. These

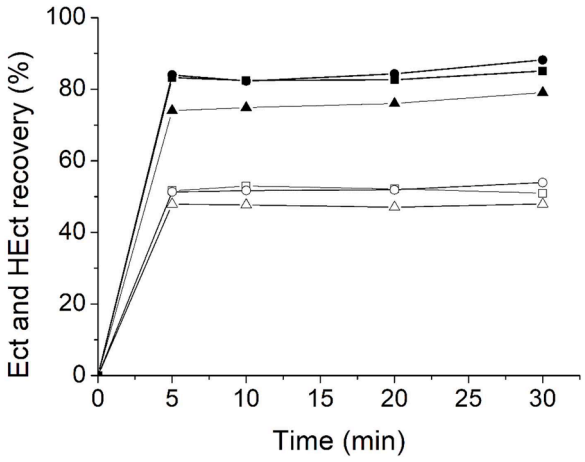

Fig. 5. Time course of ectoine (Ect, solid) and hydroxyectoine (HEct, open) recovery at different volumes of water: 10 mL (▲), 15 mL (■) and 20 mL (●), during resuspension of the biomass contained in 40 ml of culture broth.

ectoine recovery efficiencies were higher than those reported by Cantera et al. (2017b) (70±25%) using *M. alcaliphilum* 20Z incubated at 6% NaCl prior to biomass suspension in an NaCl-free medium for one hour. Similarly, Van-Thuoc et al. (2010) also reported a 90% ectoine recovery rate after subjecting *Halomonas boliviensis* cells to a hypo-osmotic shock from 15% to 0% NaCl for 30 min. The previous optimization carried out in this study obtained similar ectoine recoveries in a shorter period, thus decreasing the time and the operational costs. However, hydroxyectoine recoveries of only 51±17% and 75±16% were recorded in R2 and R3, respectively. The lower accumulation of hydroxyectoine could be the reason for the lower release of this compound compared to that of ectoine, inducing methanotrophic cells to retain higher percentages of this metabolite to survive. Overall, biomass bio-milking in R3 was more efficient in terms of ectoine and hydroxyectoine recovery (Fig. 6).

3.5. Microbial community analyses

The number of high-quality filtered reads obtained ranged between 83,491 and 187,643 (Supplementary Table 1). Rarefaction curves showed that sequence depth was sufficient to represent the bacterial diversity contained in the samples (Supplementary Fig. 1). The 16S rRNA gene sequences evidenced a diverse set of taxa, depicting different potential microbial metabolisms within the inoculum and the bioreactors. Shannon diversity index values at the species level ranged between 2.47 (R3, Stage I) and 4.67 (R2, Stage IIIb). In general, R3 showed less diverse microbial communities in Stages I and IIIb compared to R1 and R2 (Supplementary Table 2), suggesting a higher impact of bioreactor start-up and the bio-milking process on the microbial communities.

Methanotrophic bacteria were represented by members of the *Methylomicrobium* genus in all samples. *Methylomicrobium* appeared concomitantly with a variety of potential strictly aerobic heterotrophs (*Aequorivita, Legionella, Planktosalinus, Nitratireductor*) (Bowman and Nichols, 2002; Labbé et al., 2004; Taylor et al., 2009; Zhong et al., 2016) and potential aerobic and anaerobic denitrifying bacteria, including methylotrophs fed on methanol such as *Methylophaga, Hyphomicrobium* (Cucaita et al., 2021; Martineau et al., 2013) and other non-methylotrophic denitrifying bacteria likely fed on other carbon compounds such as simple sugars, fatty acids, and amino acids (*Halomonas, Oceanibaculum, Stappia, Marinobacter*) (Lai et al., 2009; Nakano et al., 2010; Villemur et al., 2019; Wang and Shao, 2021; Weber and King, 2007) (Fig. 7a). These results suggest that potential denitrifying and strictly aerobic heterotrophic bacteria detected in the bioreactors utilized carbon intermediates derived from methanotrophic metabolism and/or a bacterial lysis substrate, which is supported by previous works (Villemur et al., 2019). Even ectoine could have served as a carbon energy source within the process, since mainly heterotrophic microorganisms belonging to Proteobacteria such as *Chromohalobacter salexigens* 3043T (formerly *Halomonas elongata* DSM 3043) (Arahal et al., 2001) have developed ways to exploit ectoines as nutrients when they are no longer needed as stress protectants (Reshetnikov et al., 2020; Hermann et al., 2020; Mais et al., 2020; Vargas et al., 2006).

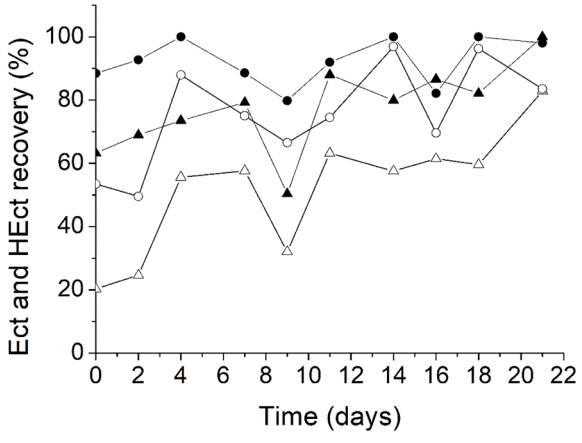

Fig. 6. Time course of ectoine (Ect, solid) and hydroxyectoine (HEct, open) recovery during the bio-milking process (stage 3b) in R2 (▲) and R3 (●).

Ectoine synthesis in R2 and R3 could potentially be attributed not only to the detected methanotroph *Methylomicrobium*, but also to other methylotrophic bacteria (*Methylophaga, Hyphomicrobium*) (Cucaita et al., 2021) and other heterotrophic organisms such as *Halomonas* with the ability to synthesize this stress protectant (Wang and Shao, 2021), all of which constitute genera detected in R2 and R3 along the different operational phases (Fig. 7a). However, the genes involved in ectoine degradation have been identified not only in *Halomonas* but also in halotolerant methylotrophs such as *Methylomicrobium* and *Methylophaga* (Reshetnikov et al., 2020). In the case of methanotrophs, ectoine degradation is used to preserve nitrogen, carbon, and energy stored in the osmolyte. For this reason, an engineered methanotrophic strain lacking the genes for degrading ectoine could be a promising approach to further increase ectoine production at an industrial scale.

Although bacteria like *Methylomicrobium* and *Stappia* were able to develop under both salinity conditions, differences in salinity (0% NaCl R1, 6% NaCl R2, R3) exerted selection pressure on the microbial communities by which certain bacteria were mainly or exclusively present in R1 (*Legionella, Oceanibaculum, Sphingomonas, Hyphomicrobium, Persicitalea, Prosthecobacter*) and others mostly or solely developed in R2 and R3 (*Methylophaga, Halomonas, Nitratireductor, Planktosalinus*) (Fig. 7b). Indeed, a cluster analysis at genus level showed that samples from R1 corresponding to Stages II, IIIa, and IIIb clustered separately from the rest of the samples at a distance of ~ 0.9, and all samples from Stage II to Stage IIIb for R2 and R3 (except R3, Stage IIIa) clustered together at a distance of ~ 0.2, indicating more similar taxa between them, likely mediated by the same salinity condition imposed on both systems (Supplementary Fig. 2).

Members of *Methylomicrobium* were present in abundances higher than 1% in all bioreactor stages, except in Stages I and IIIb in R1 (Fig. 7c, d), suggesting that low salinities along with a low CH_4 and nitrogen availability negatively affected their development. Notwithstanding, considering the abundance resulting from the sum of the *Methylomicrobium* 16S rRNA sequences in R1 and R2 (R1-R2 system) compared to the *Methylomicrobium* abundance in R3, both bioreactor configurations showed almost the same abundance of this methanotroph regardless of the operational stages (Fig. 7d). Consistent with the observed increase in the CH_4 oxidation rate at higher nitrate availabilities (Stage II) in the 3 bioreactors, the abundance of *Methylomicrobium* sequentially increased from Stage I to Stage II and to Stage IIIa in R1-R2 and R3 systems (Fig. 7c, d). Interestingly, a lower abundance of *Methylomicrobium* 16S rRNA sequences was not observed during Stage II in R2, where a lower degradation of CH_4 was recorded compared to the preceding stage. However, the lowest abundance of *Methylophaga* and *Hyphomicrobium* (two potential methanol-degrading bacteria (Cucaita et al., 2021; Martineau et al., 2013)) were detected in R2 during Stage II, suggesting a possible accumulation of methanol that could have led to methanotrophic activity inhibition without affecting *Methylomicrobium* bacterial abundance.

Overall, the bio-milking process contributed to a decrease in *Methylomicrobium* abundance in R1-R2 and R3 systems (Fig. 7c, d). However, ectoine yields remained almost constant from Stage IIIa to Stage IIIb (Fig. 3), suggesting the contribution of other members of the microbial community to the overall ectoine and hydroxyectoine yields, or the fact that *Methylomicrobium* degraded some of the ectoine produced. Despite the decrease in the abundance of *Methylomicrobium*-like species in Stage IIIb (Fig. 7c, d), no negative effect on CH_4 consumption was observed, suggesting that the modulation of the methanotrophic activity in response to stress was plausible.

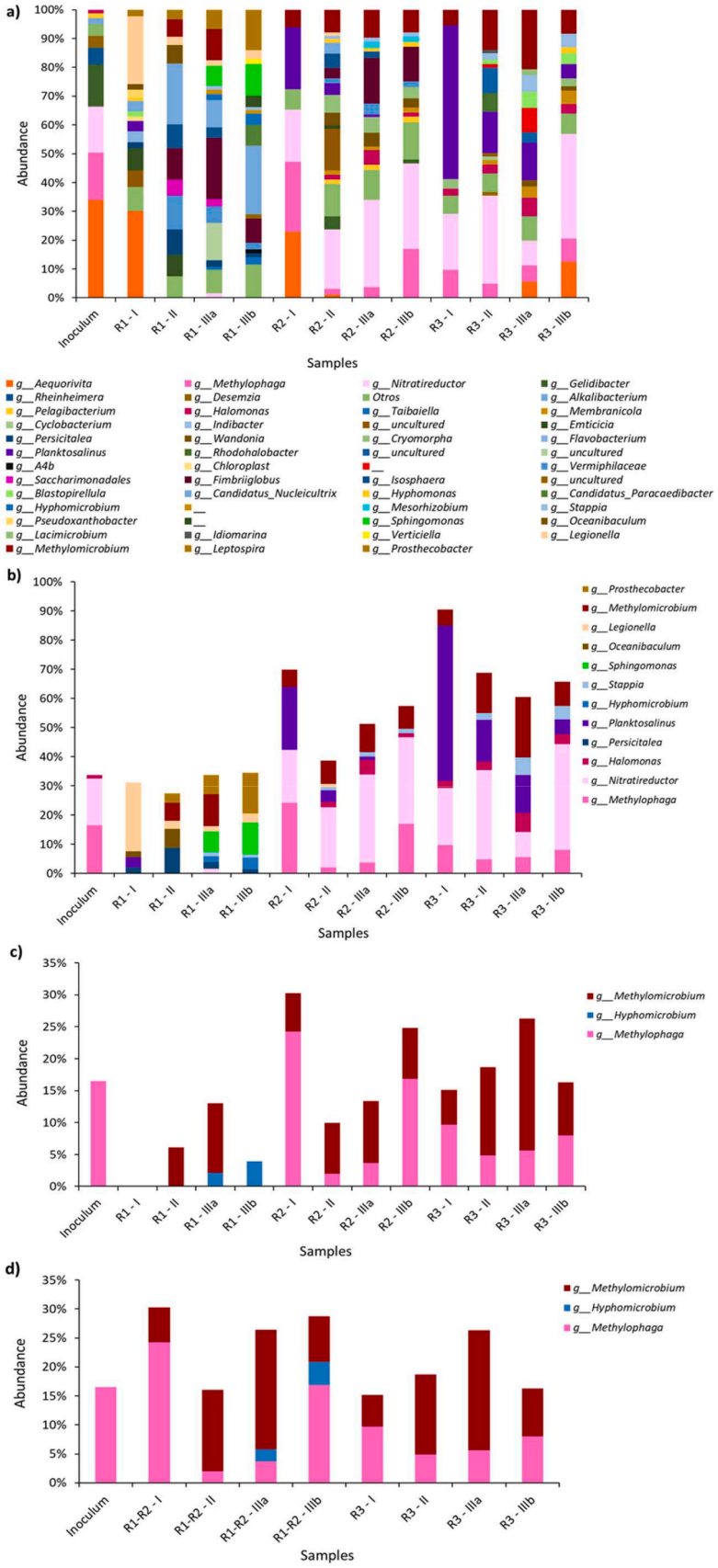

Fig. 7. Barplots of the taxonomic composition (genus level) of the samples (taxa with an abundance higher than 1% are represented): a) all genera observed in the samples, b) detail of some genera observed at both salinities (0% and 6% NaCl) and some genera exclusively present at low (R1) or high (R2, R3) salinity conditions, c) detail of *Methylomicrobium* and potential methanol-degraders (*Hyphomicrobium, Methylophaga*) in R1, R2 and R3, d) detail of *Methylomicrobium* and potential methanol-degraders (*Hyphomicrobium, Methylophaga*) in R1-R2 and R3 bioreactors.

3.6. Implications of this study

The production of ectoines using methanotrophs is a promising bioprocess that could be implemented in solid waste or wastewater treatment plants to valorize the biogas obtained from the treatment of their residues. In this sense, part of the biogas produced in the anaerobic digester could be injected into the bioreactor together with air, after biogas desulphurization, to promote methanotroph growth and ectoine accumulation under conditions of high salinity and nutrient availability. After ectoine accumulation, a certain volume of the cultivation broth could be pumped into a centrifuge to remove the saline medium and subject the methanotrophic consortium to a hypo-osmotic shock. After this downshock, a second centrifugation would be performed to separate the biomass to be recycled to the bioreactor from the ectoine broth that would be subsequently purified (Fig. 8).

This study demonstrated that a stand-alone bioreactor with an NaCl concentration of 6% is the most suitable configuration for ectoine and hydroxyectoine synthesis by a methanotrophic consortium. Due to the low ectoine concentrations usually reached by these microorganisms (maximum 109 mg/g biomass), the main objective was to improve osmolyte yields (Cantera et al., 2020). The growth of a methanotrophic consortium in a mineral medium without NaCl to initially accumulate more biomass, before subjecting part of the bacteria to hyperosmotic shock in a 6% NaCl medium to promote higher ectoine/hydroxyectoine synthesis, was a promising strategy. However, the rapid increase of salinity in the medium to a non-acclimatized culture of the former bioreactor resulted in poor efficiency in terms of CH_4 removal and ectoine synthesis in the second bioreactor. The main advantage is that the use of a stand-alone bioreactor for ectoine production will result in a decrease in the capital cost of the process at industrial scale.

The second noteworthy point of this study was the optimization of ectoine recovery via bio-milking and the subsequent recycling of biomass to the bioreactor. A previous batch study had already shown that *M. alcaliphilum* 20Z was able to respond to hypo-osmotic shocks by releasing accumulated ectoine. However, this previous work did not present bio-milking optimization, did not use a methanotrophic consortium, and did not demonstrate the feasibility of biomass recycling. It is remarkable that the release of ectoine was quick (5 min) and that the volume of water required was almost 2.7 times lower than the volume of the culture broth. These results will be very useful for a future scale-up of the process.

Here, the optimization of some operating parameters of the process in bubble column bioreactors ($N—NO_3$ required to avoid N limitation and the internal gas recirculation) was also performed. Nevertheless, more research is required to improve the process in terms of CH_4 gas-liquid mass transfer and synthesis of ectoine and hydroxyectoine. Specifically, the gas retention times must be optimized to improve the biomass yield (g biomass/g CH_4 consumed) and, as a result, ectoine yields. Even though bubble column bioreactors offer advantages such as less capital, lower operational costs, less shear stress, and easy biomass recovery, novel configurations of high mass transfer bioreactors should be also tested (Stone et al., 2017).

4. Conclusions

The continuous operation of pilot high mass transfer membrane bioreactors for the production of ectoine and hydroxyectoine from biogas was herein validated. The increase in the N loading rate and in the internal gas recirculation contributed to an increase in biomass concentration and CH_4 degradation. Nevertheless, these enhancements in methanotrophic activity did not result in higher ectoine or hydroxyectoine accumulations. The stand-alone 20 L bioreactor operating at an NaCl concentration of 6% showed a more robust performance than the two 10 L bioreactors interconnected in series at 0% and 6% NaCl, respectively. The release of ectoine to a salt-free extracellular medium was quick (<5 min) and an optimum water/cultivation broth volume ratio of 1.5/4 was identified, resulting in ectoine recoveries > 90% in R3 and a rapid acclimation of microorganisms to cyclic hypoosmotic and hyperosmotic shocks. *Methylomicrobium* was responsible for CH_4 degradation in both systems, but other potential ectoine producers were detected (*Methylophaga, Halomonas*). The high bacterial diversity observed suggests that a complex network of cross-feeding reactions occurred in the bioreactors between methanotrophs, aerobic heterotrophs, and aerobic and anaerobic denitrifying bacteria. This highlights the need to elucidate the operational factors impacting the different microbes with the ability to synthesize ectoine in systems operated using mixed consortia or an engineered methanotrophic strain in order to optimize ectoine production yields from CH_4.

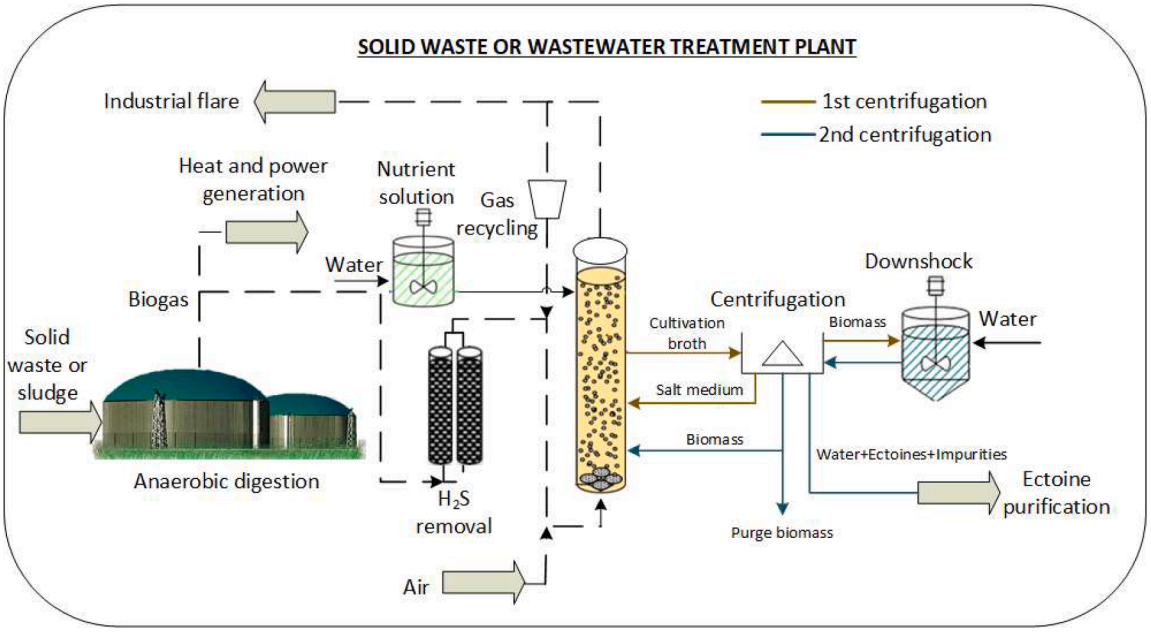

Fig. 8. Ectoines production from biogas in a solid waste or wastewater treatment plant.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Raul Munoz reports financial support was provided by European Union. Raul Munoz reports financial support was provided by Regional Government of Castilla y Leon.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.120665.

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