Bioresource Technology 444 (2026) 133920

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Influence of biomilking on methanotrophs cultivation during biogas conversion into ectoines☆

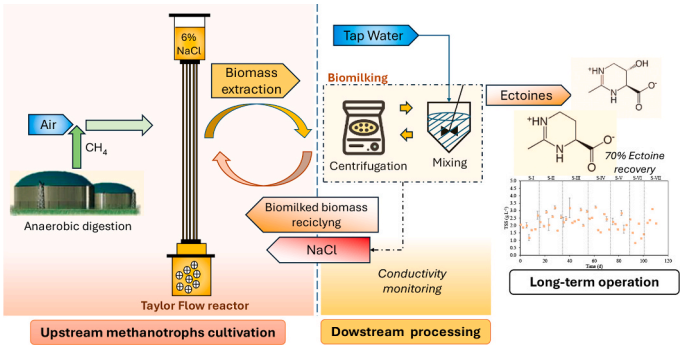
Andrés Felipe Torres-Franco, Raquel Herrero-Lobo, Raquel Lebrero, Raúl Muñoz *

Institute of Sustainable Processes, University of Valladolid, Dr. Mergelina s/n., 47011 Valladolid, Spain
Department of Chemical Engineering and Environmental Technology, School of Industrial Engineering, University of Valladolid, Dr. Mergelina s/n., 47011 Valladolid, Spain

HIGHLIGHTS

- The effect of long-term ectoines extraction on bioreactor performance was assessed.
- Ectoines extraction resulted in limited decreases in CH_4 bioconversion (~10 %).
- Extraction resulted in slight decreases in *Ect* (~2.5 %) and *Hy* (~0.5 %) content.
- Salt level control favoured *Methylomicrobium* dominance.
- Salt level control guaranteed process stability and bioconversion efficiency.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Ectoine biosynthesis
Ectoine extraction
Methane bioconversion
Taylor Flow reactor

ABSTRACT

Ectoine is one of the most profitable value chains for biogas valorisation. This study assessed the long-term effects of biomilking for ectoine (*Ect*) and hydroxyectoine (*Hy*) extraction on upstream methane bioconversion into ectoines using a halotolerant methanotrophic consortium cultivated in a Taylor-Flow bioreactor. After a control stage (S-I), fractions of the culture broth volume of 10 %, 50 %, 30 % and 60 % (S-II to S-VI) were subjected to biomilking before a final control (S-VII). No adverse effects were observed at 10 %, while higher fractions led to salt depletion, a ~10 % reduction in CH_4 bioconversion efficiency, and a loss of dominant ectoine producers, mainly *Methylomicrobium*. A decrease in intracellular *Ect* was also observed. Restoring salt levels (S-VI) recovered *Methylomicrobium* dominance and *Ect* content in the culture broth. Enhanced biomilking yielded up to 27.2 g-*Ect* + *Hy* per inlet kg of CH_4, supporting its feasibility for sustainable biogas valorisation at a commercial scale.

☆ This article is part of a special issue entitled: 'IWA ecoSTP2025' published in Bioresource Technology.

* Corresponding author at: Institute of Sustainable Processes, University of Valladolid, Dr. Mergelina s/n., 47011 Valladolid, Spain.
E-mail addresses: andresfelipe.torres@uva.es (A.F. Torres-Franco), raquel.herrero.lobo@uva.es (R. Herrero-Lobo), raquel.lebrero@uva.es (R. Lebrero), raul.munoz.torre@uva.es (R. Muñoz).

https://doi.org/10.1016/j.biortech.2026.133920
Received 21 October 2025; Received in revised form 29 December 2025; Accepted 1 January 2026
Available online 2 January 2026

1. Introduction

The rapid decrease in the cost of renewable energies, such as solar or wind power, has encouraged the research of new alternatives for biogas valorisation, including its bioconversion into high-value-added products. Consequently, the anaerobic digestion of biowastes has shifted from biogas on-site application for heat or electricity to more innovative biorefinery concepts, where added-value compounds can be extracted from wastes (Fermoso et al., 2018). With a market price of 600–1000 €·kg^{-1} (Pérez et al., 2021), ectoine and hydroxyectoine are examples of high-value compounds that can be produced on innovative platforms for valorising biogas and CO_2 obtained during the anaerobic treatment of waste streams (Rodero et al., 2022; Huang-Lin et al., 2024). These osmolytes are synthesised by certain halophilic and halotolerant microorganisms to maintain osmotic balance under high-salinity stress conditions (Ng et al., 2023). Their high value in pharmaceutical and cosmetic applications derives from their ability to counteract oxidative and heat stress, protect enzymes, DNA, and entire cells, and support overall cellular stability (Pastor et al., 2010). Reported uses include dermatological cosmetic additives and potential therapeutic applications for Alzheimer's disease, HIV, intestinal and oral diseases, and various inflammatory and immunological disorders, among others (Chen et al., 2024).

While several genera can produce ectoine, the ability to naturally synthesise both ectoine and hydroxyectoine is more restricted, being reported in taxa such as *Actinomycete, Bacillus*, *Halobacillus*, *Halomonas, Micrococcus, Nesterankonia*, and *Thalassobacillus* (Severin et al., 1992; Bursy et al., 2007; Orhan and Ceyran, 2023). Triggered by medium salinity, the biosynthetic pathway of these compatible solutes is based on the *ectABC* gene cluster, which converts the precursor metabolite L-aspartate-ß-semialdehyde (ASA) into ectoine, which is then hydroxylated to hydroxyectoine by the ectoine hydroxylase (*Ect*D) (Louis and Galinski, 1997; Bursy et al., 2007). Current ectoine and hydroxyectoine production platforms are mainly based on batch fermentations at high salt concentrations, using mainly *Halomonas* (e.g., Chen et al., 2017) or genetically engineered *Halomonas* or *E. coli* (e.g., He et al., 2015; Tanimura et al., 2016), on relatively expensive carbon and nitrogen sources such as glucose, glutamate, glutamine, or yeast extract (Pastor et al., 2010; Chen et al., 2024). As an alternative to sugar fermentation, bacterial platforms using residual greenhouse gases have been researched based on methanotrophic haloalkaliphilic bacteria (Cantera et al., 2017, 2017b), or axenic cultures such as *Hydrogenbacillus schlegelii* (Marcos-Rodrigo et al., 2025) and *Hydrogenovibrio marinus* (Huang-Lin et al., 2024).

Mixed methanotrophic consortia can also be cultured in waste gas streams for ectoines production, such as that in Carmona-Martínez et al. (2021). In this case, the process performance was previously investigated in different bioreactor configurations, such as bubble column (Rodero et al., 2023, 2024) or Taylor flow bioreactors (Herrero-Lobo et al., 2024, 2025), achieving methane bioconversion efficiencies of up to 65.5 % and 89.2 %, and maximum intracellular ectoine content of 79 mg-Ect gVSS^{-1} and 105 mg-Ect gVSS^{-1}, respectively. Mixed methanotrophic consortia offer several benefits, including increased biomass, enhanced biocatalytic performance, and greater system stability. In these consortia, heterotrophic bacteria can supply essential nutrients, mitigate culture toxicity, stimulate methanotroph growth and activity and produce biologically active growth-promoting compounds (Singh et al., 2019).

Regardless of the cultivation strategy, the process must be coupled with an extraction step, which is required for subsequent downstream purification. One alternative for this extraction is cell lysis via acid treatment at moderately high temperatures (60 °C-70 °C), operating the process as fed-batch cultivation and constantly growing the cells at the beginning of each cultivation cycle (Chen et al., 2017). Another strategy involves subjecting the halotolerant culture to a hypoosmotic shock, releasing ectoines into the new low-salinity medium, and concomitantly recovering the biomass through centrifugation. Later, the biomass can be resuspended again in the original mineral salt medium and recirculated to the bioreactor. This process, known as bacterial milking or biomilking (Sauer and Galinski, 1998), is the most common across all cultivation platforms. The hypoosmotic shock is typically induced with tap water, and the milked biomass can be resuspended in the mineral medium that was separated during the first centrifugation cycle. At this point, the ectoines solution can be transferred to the downstream process, where it is purified from remaining salts and cell debris, and subsequently concentrated into a final precipitate (Ng et al., 2023). After biomilking, ectoine purification typically involves desalting, ion-exchange chromatography, and crystallisation (Pérez et al., 2021).

Despite the advantage of biomass recovery and recycling, few studies have examined how coupling biomilking affects methane bioconversion capacity and ectoine accumulation by halotolerant cultures. Among these studies, Cantera et al. (2017b) achieved excretion efficiencies of 70 % of the total intracellular ectoine accumulated by *M. alcaliphilum* 20Z under continuous cultivation in two sequential stirred-tank reactors operated at NaCl concentrations of 0 and 6 %. Similarly, Lee et al. (2022) achieved up to 126 mg-*Ect* per gram of *M. alcaliphilum* 20Z *DP2* biomass and more than 90 % efficiency with biomilking in a medium containing 1 % NaCl. Finally, Rodero et al. (2023) also performed biomilking of a mixed methanotrophic consortium cultivated in a bubble column bioreactor. The process was based on two centrifugation cycles and achieved recoveries above 70 % of the intracellular ectoine, with no negative impact on methanotrophic cell recycling when 15 % of the culture broth was daily extracted for biomilking. Despite the promising results obtained by implementing continuous biomilking during biogas bioconversion into ectoines, the fractions of culture broth subjected to biomilking in those studies were relatively low. Additionally, the impact of fractions of culture broth subjected to biomilking beyond 30 % on the upstream process and on long-term operation has not been assessed to date.

Considering that intensifying ectoines extraction is critical to enhance the economic sustainability of ectoine platforms for biogas valorisation, this work evaluated the impact of long-term biomilking operation at high biomilking rates of up to 60 % on ectoines production and biogas bioconversion efficiencies by a mixed methanotrophic halotolerant consortium in a high-mass transfer Taylor flow reactor. The effect of salt supplementation for enhanced cultivation, when coupled with biomilking, was additionally assessed.

2. Materials and methods

2.1. Inoculum, mineral salt medium and gas feed

The inoculum corresponded to a halotolerant methanotrophic consortium originally enriched by Carmona-Martínez et al. (2021) and used in previous studies (Herrero-Lobo et al., 2024, 2025). This inoculum, previously acclimated to the Taylor flow bioreactor (TFB), was selected for its intrinsic resilience of the process from a microbiological perspective and its non-requirement for sterile conditions (Carmona-Martínez et al., 2021). The inoculum, cultivated as described in Herrero-Lobo et al. (2025), was mainly dominated by ectoines producers such as *Methylomicrobium, Methylophaga*, and *Paracoccus;* along with other genera such as *Nitratireductor*, and *unidentified_Saprospiraceae* when transferred to the bioreactor. To trigger ectoine production, the liquid medium of the reactor consisted of a mineral salt medium (MSM) with 6 % w/w NaCl, prepared as described in Herrero-Lobo et al. (2024) and containing all required macro and micronutrients. All reagents were purchased from Panreac Applichem (Spain) and COFARCAS (Spain) with a purity of > 99 %. Methane at 4.8 % v/v in air was fed from a 50 L cylinder (99.5 % CH_4, Carburos Metálicos S.A., Spain). Dehumidified CO_2-free air was provided by a SMART 18 compressor (SCC, Spain).

2.2. Experimental set-up

The experimental unit (see Supplementary Material) consisted of a TFB with a working volume of 7.4 L and 25 glass capillaries (h = 1.5 m, D = 3 mm). These capillaries enabled the interconnection of the upper (6.5 L) and lower (2.5 L) modules, both of which were built in polyvinyl chloride (PVC). The CH_4-laden air was fed into the lower module of the TFB, which was filled with Kaldnes® rings (Evolution Aqua, UK) to improve gas–liquid mixing and achieve homogeneous dispersion in the capillaries. The liquid media and gas bubbles flowed through the glass capillaries at flow rates of 5.2 and 5.5 L/min, resulting in linear velocities of approximately 0.5 m/s, which guaranteed Taylor flow and high gas–liquid transfer rates, as previously characterised by a hydrodynamic analysis of the same experimental unit (Cattaneo et al., 2022; Kraakman et al., 2023). The upper module facilitated gas–liquid separation through a perforated basket, which was installed for the recirculation of the liquid media. The gas outlet was divided into the gas recirculation and the off-gas lines. The gas recirculation was pumped by a membrane compressor (H5P3 EAD, Spain) after passing through a water trap and a condenser with a water jacket (4 °C) to remove humidity. Before entering the lower module, the recirculated gas was mixed with the inlet gas in a mixing chamber. The mixture of inlet air and CH_4 was prepared in a previous mixing chamber, where air was fed at a controlled pressure of 0.8 bar and CH_4 injection was controlled by a mass flow controller (AalborgTM, USA). The flow rates in the inlet and recirculation lines were controlled using gas rotameters (AalborgTM, USA) and monitored through gas sampling ports, which also served as installation points for pressure sensors (IFM PN7097). The culture broth was recirculated from the upper to the lower module by means of a centrifugal pump (Oase, EDEN 159), followed by a liquid rotameter (Fischer & Porter Iberica, 10A1197A).

Biomilking was carried out using a Thermoscientific™ X pro series or a Thermoscientific™ Lynx4000 centrifuge. Biomilking was performed daily by semi-batch extractions of ectoine (*Ect)* and hydroxyectoine (*Hy)* from variable fractions of the culture broth volume (V_R), ranging from 10 %·V_R to 60 %·V_R. Each biomilking cycle began with the manual extraction of the corresponding culture broth volume, which was then centrifuged to separate the biomass (10,000 × g, 10 min). The biomass pellet was resuspended in ultrapure water at a ratio of 1:4 (water:culture broth), agitated for 5 min in a plastic vessel (2 L) at about 700 rpm using a magnetic stirrer (Fisherbrand SP88857200) to improve the release of the intracellular content of ectoines to the water medium (hypoosmotic shock), and then subjected to a second centrifugation to separate the biomass from the ectoines solution (product). Then, the biomass pellet was resuspended in the mineral medium, which had been previously reserved in a plastic vessel (2 L) after the first centrifugation, and returned to the reactor.

2.3. Experimental design

The TFB was operated under conditions of high CH_4 bioconversion capacity at moderate efficiencies (Herrero-Lobo et al., 2025), with a gas retention time (GRT) of 30 min, total suspended solids concentration of ~2.0 g L^{-1} and the above-mentioned liquid and gas recirculation flow rates (Q_L and Q_G). Fresh MSM was added to the culture broth at a rate of 740 mL d^{-1} (10 % of the working volume) by extracting and centrifuging the same volume of culture broth, resuspending the biomass in fresh MSM, and then transferring it to the TFB. The TFB was operated for 110 days under daily biomilking and recirculation of the culture broth after ectoines extraction. The operational plan is summarised in Table 1. A first and a last control stage without biomilking (S-I and S-VI) allowed to respectively assess the CH_4 bioconversion efficiencies and capacities before biomilking operation and the systems recovery after prolonged periods of osmolytes extractions. During S-II and S-III, the Thermoscientific™ X pro series was used for biomilking, while the Thermoscientific™ Lynx4000 centrifuge was used from S-IV onwards in order to improve centrifugation efficiencies at higher culture broth volumes. Besides MSM renewal, during S-VI, biomilking was performed at 60 % with ionic conductivity correction by supplementing NaCl. This correction was done before returning the biomilked biomass to the TFB at approximately 20 g-NaCl per litre of culture broth per day during the first three days of this stage.

Table 1
Elapsed time and biomilking volumes per operational stage.

Stage	Elapsed time (days)	Culture broth volume subjected to biomilking (L)	Fraction of culture broth biomilked (%)
S-I	14	–	–
S-II	19	0.7	10 %
S-III	21	3.7	50 %
S-IV	18	2.2	30 %
S-V	14	4.4	60 %
S-VI	12	4.4	60 % (NaCl)
S-VII	12	–	–

The monitoring of the inlet and outlet gas composition, as well as of the liquid phase in the culture broth and the biomilking product solution, was performed three times a week. A 100 μL gas-tight syringe (Hamilton, USA) was used to collect duplicate samples from the inlet and outlet gas streams and assess concentrations of CO_2, N_2, O_2 and CH_4 by Gas Chromatography (GC-TCD). Culture broth samples were withdrawn to analyse pH, conductivity, and the concentrations of total suspended solids (TSS), dissolved total organic carbon (TOC), inorganic carbon (IC) and total nitrogen (TN), and intra-cellular *Ect* and *Hy*. Furthermore, the DNA from culture broth samples was extracted and sent for next-generation sequencing (NGS) of 16S rRNA. Samples of the biomilking solution (ultrapure water containing *Ect* and *Hy*) were analysed for *Ect* and *Hy* concentrations, pH, conductivity and TOC and TN content.

To prevent the accumulation of inhibitors and ensure the availability of micro and macronutrients, 10 % of the cultivation broth in the TFB was centrifuged and replaced daily with fresh mineral medium. Aliquots of culture broth were periodically withdrawn in order to maintain TSS concentration at ~ 2 g L^{-1}. Conversely, when TSS concentrations were lower than the setpoint, the biomass centrifuged with the daily renewal of mineral medium was resuspended in fresh media and returned to the reactor.

2.4. Analytical procedures

The inlet and outlet gas flow rates were determined by water displacement in an inverted measuring cylinder. The inlet flow rate was measured using a bypass line equipped with a needle valve to adjust the pressure in the bypass to match that in the system. Before the gas flow rate measurements, concentrations of CO_2, O_2, N_2, and CH_4 were determined by gas chromatography (Bruker 430, Palo Alto, USA) equipped with a thermal conductivity detector and a CP-Molsieve 5A and a CP-PoraBOND Q columns. The oven, injector and detector temperatures were maintained at 45, 150 and 200 °C, respectively, for 5 min.

Ionic conductivity and pH in the culture broth and biomilking solutions were measured with an EC-Meter BASIC 30 instrument (Barcelona, Spain) and a Basic 20 + pH-meter (Düsseldorf, Germany), respectively. A TOC-VCSH analyser (Japan) interconnected with a TNM-1 chemiluminescence module was used to measure TOC, TN and IC concentrations in samples filtered through 0.45 μm. TSS concentrations in the culture broth were determined following the procedure described in Herrero-Lobo et al. (2024).

Concentrations of *Ect* and *Hy* in the culture broth were determined by washing twice the biomass from 2 mL samples with a 6 % NaCl solution prepared in ultrapure water. Then, samples were centrifuged again (9 000 × g for 10 min), the supernatant was discarded, and the biomass was resuspended in ethanol (70 % w/w) containing 25 ± 5 mg of 0.1 mm-

diameter zirconia/silica beads, and agitated and homogenised in a Mini-BeadBeater-16 (BioSpec, Spain) at 1048 × g for 10 min. Samples were centrifuged again to separate the ethanol solution containing the *Ect* and *Hy* released from the cells after bead-beating, and filtered through 0.22 μm pore size filters. Intracellular *Ect* and *Hy* were estimated by dividing the culture broth concentrations by the corresponding TSS concentrations. On the other hand, 2 mL samples were collected and filtered through 0.22 μm pore size filters for the determination of the *Ect* and *Hy* concentration in the biomilking product solution. All samples were frozen-stored until analysis. All *Ect* and *Hy* samples were determined using an HPLC-V 717 plus autosampler (Waters, Bellefonte, USA) coupled to a UV Dual λ Absorbance detector (Waters, Bellefonte, USA) operated at 220 nm and 40 °C. As described by Carmona-Martinez et al. (2021) and Herrero-Lobo et al. (2024), an LC-18 AQ þ C Supelcosil column (Sigma Aldrich, Spain) and a C18 AQ + pre-column (Sigma Aldrich, Spain) were used. The mobile phase consisted of ultrapure water at a flow rate of 0.6 mL min^{-1}. Biomilking product samples were analysed using a Spherisorb Amino (NH_2) Column, 80 Å, 3 μm, 4.6 mm × 150 mm (Waters, USA), with Acetonitrile:Water, 75 %:25 % (v/v) as the mobile phase (0.6 mL·min^{-1}). Standards for quantification were prepared with commercial *Ect* and *Hy* (purity ≥ 95 %, Sigma Aldrich) diluted in ethanol 70 %v/v or ultrapure water. All analyses were done in technical duplicates.

2.5. Bacterial community analyses

The structure of the bacterial community in the different operational stages was determined by NGS. For DNA extraction, 1.5 mL of culture broth samples were obtained by pooling three 0.5 mL samples collected by the end of each steady state. Pooled samples were centrifuged at 13,000 × g, and the biomass pellet was used for DNA extraction using the PowerSoil Pro kit (Qiagen, Germany) following the manufacturer's instructions. A Qubit 4 Fluorometer (Thermo Fisher Scientific, USA) and Qubit 1X dsDNA HS reagents were used to measure the final DNA concentrations. The DNA extracts were sent to Novogene (UK) for Next Generation Sequencing of 16S rRNA genes of distinct regions (16SV4/16SV3/16SV3- V4/16SV4- V5, 18SV4/18SV9, ITS1/ITS2, ArcV4) on Illumina platform. Quality control was done between each step of the procedure. PCR amplification of targeted regions was performed by using specific primers connecting with barcodes, ligated with Illumina adapters and sequenced on the Illumina paired-end platform. Paired-end reads were merged using FLASH (V1.2. 1 1). Quality filtering on the raw tags was performed using the FASTP (Version 0.23.1) software. Silva databases and the VSEARCH package (V2.16.0) were used for Chimera removal. The DADA2 method was used to denoise clean data and obtain ASVs (Amplicon Sequence Variants). Species annotation was performed using QIIME2′s (Version QIIME2–202202), with the Silva 138.1 database for 16S/18S. The Unite v9.0 database 147 was used for ITS, while the micro_NT database (a curated subset of the 148 NT database containing archaea, fungi, viruses, and bacteria) was used for unconventional regions. The clean sequences were submitted to NCBI BioSample (PRJNA1346682).

2.6. Data treatment and statistical analyses

The CH_4 bioconversion efficiency (BE), CH_4 bioconversion capacity (BC), volumetric CO_2 production (PCO_2) and CO_2 production yield (Y_{CO_2}) were calculated according to Herrero-Lobo et al. (2025), following Eq. (1) to (4). Furthermore, the efficiency of intracellular *Ect* and *Hy* extraction and the ectoines (Y_{Ect+Hy}) production yield in terms of inlet CH_4 were calculated using Eq. 5 and 6:

$$CH_4 - BE\,(\%) = \frac{m_{CH_4,\,in} - m_{CH_4,\,out}}{m_{CH_4,\,in}} \times 100 \quad (1)$$

$$CH_4 - BC\left(\frac{gCH_4}{m^3 h}\right) = \frac{m_{CH_4,in} - m_{CH_4,out}}{V_R} \quad (2)$$

$$P_{CO_2}\left(\frac{g\,CO_2}{m^3\,h}\right) = \frac{m_{CO_2,out} - m_{CO_2,in}}{V_R} + \frac{Q_{L,\,MSM}\left(IC_{L,\,out} - IC_{L,\,in}\right)}{V_R} \times \frac{MW_{CO_2}}{MW_C} \quad (3)$$

$$Y_{CO_2}\left(\frac{g\,CO_2}{g\,CH_4}\right) = \frac{P_{CO_2}}{CH_4 - BC} \quad (4)$$

$$Ectoines\ \ extraction\ \ efficiency\,(EE) = \frac{X_{PS}V_{PS}}{X_{CB}V_B}\,(\mathrm{EE}) \quad (5)$$

$$Y_{Ect+Hy}\left(\frac{kg\,Ect + Hy}{kg\,inlet\,CH_4}\right) = \frac{\left(X_{Ect} + X_{Hy}\right)V_{PS}}{m_{CH_4,\,in}} \quad (6)$$

where, $m_{CH_4,in}$ and $m_{CH_4,out}$ correspond to inlet and outlet mass flow rates of methane (g-CH_4 h^{-1}). Similarly, $m_{CO_2,in}$ and $m_{CO_2,out}$ are the inlet and outlet mass flow rates of CO_2 (g-CO_2 h^{-1}). In Eq. (3), $Q_{L,MSM}$ is the fresh MSM exchange flow rate, $IC_{L,IN}$ and $IC_{L,OUT}$ correspond to the inorganic carbon concentrations (g-IC L^{-1}) in the fresh MSM and in the culture broth, respectively, while MW_{CO2} and MW_C stand for the molecular mass of CO_2 and carbon. In Eq. (4), X_{PS} correspond to the *Ect* or *Hy* concentrations in the biomilking product solution (PS) and V_{PS} is the volume of the PS, while X_{CB} and V_B correspond to the *Ect* or *Hy* concentrations in the culture broth and the volume of culture broth devoted to biomilking.

A Kruskall Wallis test followed by a Dunńs test post hoc was applied to detect significant differences between operational stages. Spearman correlations were applied to explore associations between bacterial population abundances and ectoine content. Data treatment and statistical test were performed in Rstudio environment and Past 5.2.1.

3. Results and discussion

3.1. Influence of biomilking on methane bioconversion

Fig. 1 shows the evolution of CH_4-BE, CH_4-BC, and CO_2 yield (Y_{CO2}) as a function of the culture broth volume subjected to daily biomilking for ectoines extraction. The average CH_4-BE and CH_4-BC in the control Stage I without biomilking were 42.2 % ± 3.4 and 26.9 ± 3.5 g-CH_4 $m^{-3}h^{-1}$, respectively. No significant decreases in CH_4-BE and CH_4-BC compared to S-I were observed during daily ectoine extraction through biomilking at 10 %·V_R (S-II) and 50 %·V_R (S-III). However, significantly lower average CH_4-BE and CH_4-BC were observed from S-IV to S-VI, particularly in S-VI, when performance dropped to 33.6 % ± 2.2 and 23.1 ± 2.5 gCH_4 $m^{-3}h^{-1}$, respectively. During S-VII, biomilking was stopped, and CH_4-BE and CH_4-BC recovered similar levels to those observed during S-I, averaging 39.4 % ± 3.7 and 27.6 ± 2.2 g-CH_4 $m^{-3}h^{-1}$. The CO_2 yield confirmed methanotrophic activity throughout the operational period, with mean values ranging from 1.4 to 1.8 g-CO_2 g-CH_4^{-1}, and no significant decreases during biomilking operation (S-II to S-VI) compared to control stages (S-I and S-VII).

The CH_4-BE and CH_4-BC averages achieved during the control stages (S-I and S-VII) were slightly lower than those achieved by Herrero-Lobo et al. (2025) in the same experimental unit at 30 min of GRT and 5 % inlet CH_4 concentration (47.0 % ± 1.5 and 33.0 ± 2.7 g-CH_4 $m^{-3}h^{-1}$). These slight differences were likely due to the lower average inlet CH_4 concentrations of 4.8 % used in this study. This setpoint allowed the evaluation of inlet concentrations below 5 %, ensuring higher ATEX security in complex industrial applications. Compared to previous studies with methanotrophs cultivation in both Bubble Column and Taylor flow bioreactors, higher efficiencies were facilitated mainly by lower salinity levels (*e.g* 73 % in García-Pérez et al., 2018), and higher GRT (*e.g* 79 % at 60 mins in Rodero et al., 2023; up to 73 % at 54 mins in Rodero et al., 2024; and 50 % at 60 mins in Cattaneo et al., 2022).

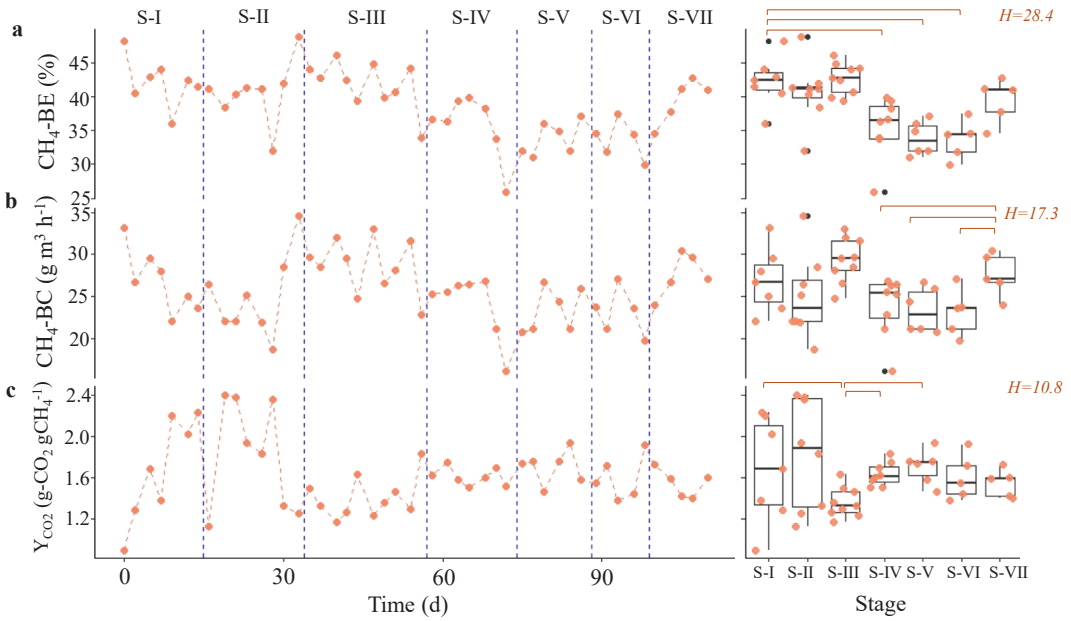


Fig. 1. Time course and boxplot of (a) Methane bioconversion efficiency (CH_4-BE, %), (b) Methane bioconversion capacity (CH_4-BC, g m^{-3} h^{-1}), and (c) CO_2 production yield (Y_{CO_2}, gCO_2 gCH_4^{-1}). Breaks in the boxplot figures indicate the most relevant significant pairwise comparisons in Dunn's test.

Despite the gas–liquid methane mass transfer limitations, the results achieved during S-I validated the high gas–liquid transfer of the TFB, even at the relatively short GRT of 30 min, which was adopted to enhance the total mass of CH_4 bioconverted, albeit at the expense of moderate efficiencies.

No significant decreases in CH_4-BE and CH_4-BC were observed during continuous biomilking of 10 % of the culture broth (S-II), which agreed with results from previous works at similar biomilking rates in a bubble column bioreactor (Rodero et al., 2023) or in a stirred tank reactor (Cantera et al., 2017b). To the best of our knowledge, no previous works have assessed the impact of biomilking rates above 30 % of the culture broth, which limits direct comparison of the results achieved at 50 % V_R (S-III) with those reported in the literature. At this stage, the negligible effects of continuous biomilking on CH_4-BE and CH_4-BC were likely due to the absence of stress conditions in the methanotrophic metabolism during previous stages. However, the higher biomilking rate of 50 % applied in S-III appeared to disturb the culture broth, resulting in significant decreases during subsequent biomilking at 30 % and 60 % (S-IV to S-V). These decreases were most likely caused by the cumulative stress of prolonged biomilking, which by the end of S-III had already exceeded 40 days of continuous ectoines extraction, slowing metabolic rates, including CH_4 oxidation (Cantera et al., 2017). The addition of NaCl in S-VI induced no enhancement in CH_4-BC and CH_4-BE, while stopping biomilking during S-VII resulted in a rapid recovery of methane removal efficiencies. Despite the observed variations in CH_4-BC and CH_4-BE, methanotrophic activity throughout all stages was validated by

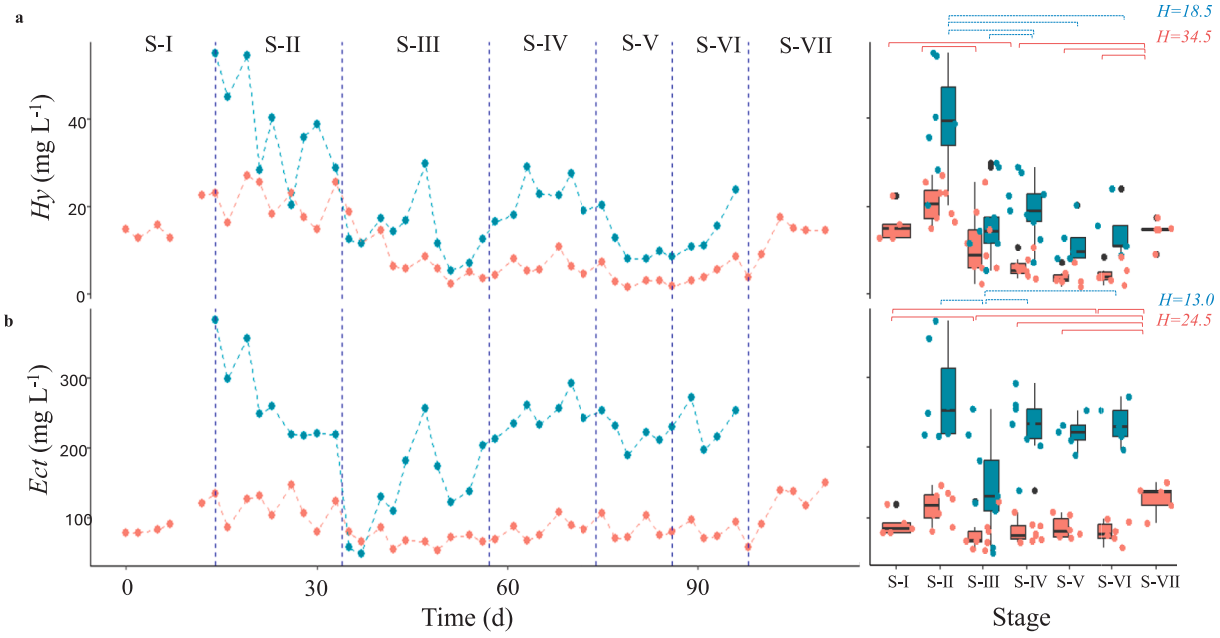


Fig. 2. Time course and boxplots for (a) Hydroxyectoine (*Hy*) and (b) Ectoine (*Ect*) concentrations in the culture broth (●) and in the biomilking product solution (●). Breaks in the boxplots figures indicate the most relevant significant pairwise comparisons in Dunńs test.

the CO_2 production yields, which were comparable to the values of 1.7 to 2.3 g-CO_2 g-CH_4^{-1} reported by Herrero-Lobo et al. (2025) during TFB operation at 30 min GRT with diluted methane emissions.

3.2. Influence of biomilking rate on ectoine production and extraction

During control stages S-I and S-VII, the bioconversion of biogas into *Ect* and *Hy* resulted in average concentrations of these osmolytes in the culture broth of 97.9 ± 21.8 mg-*Ect* L^{-1} and 127.2 ± 20.8 mg-*Ect* L^{-1}, and 17.0 ± 4.2 mg-*Hy* L^{-1} and 14.1 ± 2.8 mg-*Hy* L^{-1}, respectively (Fig. 2). The culture broth was characterised by TSS concentrations of 2.2 ± 0.5 g L^{-1} throughout the experiment (see Supplementary Material), which resulted in average intracellular contents of 52.7 and 58.5 mg-*Ect* $gTSS^{-1}$, and 9.0 and 6.4 mg-*Hy* $gTSS^{-1}$ in control stages S-I and S-VII, respectively (see Supplementary Material). These results validated the capacity of the TFB to support an active growth of ectoine-accumulating methanotrophic bacteria at relatively high biomass concentrations.

Process biomilking at 10 % (S-II) resulted in no significant decreases in osmolytes concentrations or intracellular contents compared to control stages, which were only observed at higher biomilking rates (S-III to S-VI). Interestingly, S-III showed the lowest average concentrations of ectoines in the culture broth (69.2 mg-*Ect* L^{-1} and 8.8 mg-*Hy* L^{-1}) and intracellular contents (27.9 mg-*Ect* $gTSS^{-1}$ and 3.5 mg-*Hy* $gTSS^{-1}$), corresponding to reductions of ~ 16 % and 8 % in the *Ect* and *Hy* intracellular content. The addition of NaCl to restore the salinity in the cultivation broth during S-VI increased the intracellular *Ect* content to 53.8 mg $gTSS^{-1}$, without significant effects on the *Hy* content or on the culture broth concentrations of both osmolytes. Initial culture broth concentrations of *Ect* and *Hy* were recovered only when biomilking was stopped during S-VII (Fig. 2).

The gradual loss of salinity due to continuous biomilking of the culture broth, along with stress from long-term daily extractions, was likely the main factor behind the decreases in ectoines concentrations and intracellular contents. Regardless of the decrease in ectoines accumulation mediated by continuous biomilking, the methanotrophic consortia showed similar ectoines accumulation capacities to previous experiments: 20–52 mg-*Ect* $gVSS^{-1}$ (Rodero et al., 2023), 45–79 mg-*Ect* $g\text{-}VSS^{-1}$ (Rodero et al., 2024), 37–49 mg-*Ect* $g\text{-}VSS^{-1}$ and 8 to 13 mg-*Hy* $g\text{-}TSS^{-1}$ (Herrero-Lobo et al., 2025).

The product solution from biomilking exhibited average osmolytes concentrations ranging from 144.3 mg-*Ect* L^{-1} to 274.7 mg-*Ect* L^{-1}, and from 16.4 mg-*Hy* L^{-1} to 39.7 mg-*Hy* L^{-1}, with the lowest and highest average concentrations of *Ect* recorded at S-III and S-II, respectively. From S-IV to S-VI, *Ect* and *Hy* concentrations increased to relatively similar levels to those recorded in S-II, averaging 229.4 mg-*Ect* L^{-1} and 17.8 mg-*Hy* L^{-1} (Fig. 2). These concentrations corresponded to increasing productivities of 71.4 mg-*Ect* (S-II) to 312.8 mg-*Ect* (S-VI) and 10.3 mg-*Hy* (S-II) to 18.2 mg-*Hy* (S-VI) per cycle of biomilking, and extraction efficiencies of 62.0 % and 63.7 % for *Ect* and 50.1 % to 72.8 % for *Hy* during the same operational stages (Table 2). These same productivities expressed per gram of VSS subjected to biomilking were of 29.8 ± 1.7 mg-*Ect* (S-II) to 39.2 ± 14.4 mg-*Ect* (S-VI) and of 4.4 ± 1.7 mg-*Ect* (S-II) to 2.3 ± 1.1 mg-*Ect* (S-VI).

Biomilking with axenic cultures has demonstrated high extraction efficiencies. *H. boliviensis* achieved 75 % efficiency and productivities of 9.1 g L^{-1} d^{-1} (ectoine) and 2.0 g L^{-1} d^{-1} (hydroxyectoine) at 15 % NaCl (Van-Thuoc et al., 2010), while *H. elongata* reached 64 % efficiency and 155 mg $g\text{-}CDW^{-1}$ (Sauer & Galinski, 1998). Fed-batch acid extraction achieved up to 76 % efficiency at 60–70 °C (Chen et al., 2017). Similarly, Lee et al. (2022) achieved more than 90 % efficiency during the biomilking of a culture of *M. alcaliphilum* 20Z DP2. Although these values exceed those obtained in the present study, they relied on synthetic media with costly substrates (e.g., glutamine) and were performed at lab scale with short volumes (<2 L).

In contrast, Cantera et al. (2017b) and Rodero et al. (2023) integrated biomilking with the conversion of methane from biogas as the sole carbon and energy source. Cantera et al. (2017b) reported ~ 70 % ectoine extraction from *M. alcaliphilum* 20Z in a two-stage system, yielding 253.4 ± 55.1 mg L^{-1} with ~ 20 % of the broth biomilked. Rodero et al. (2023), using a mixed consortium and biomilking of 15 % of a 20 L reactor, achieved 83 % and 52 % extraction efficiencies for *Ect* and *Hy*, respectively, producing 224 mg L^{-1} and 33 mg L^{-1} in the final solution. Despite moderate extraction efficiencies in the present work (50–64 % for ectoine; 42–78 % for hydroxyectoine), associated mainly to higher culture volumes and biomilking fractions (10–60 %) herein tested, the concentrations of *Ect* and *Hy* achieved were comparable to those reported by Cantera et al. (2017b) and Rodero et al. (2023), validating the potential of the one-step cultivation in the TFB using a mixed methanotrophic for biogas valorisation into ectoine.

Regarding biomilking performance, the concentrations in the biomilking product solution, ~ 3-fold those in the culture broth, were slightly below the 1:4 water-to-culture broth ratio used for resuspending the biomass during biomilking. In this sense, the product volumes (Table 2) were higher than expected due to limitations in the manual separation of biomass pellets and supernatant after each centrifugation cycle, particularly when using the Thermoscientific™ X pro series. Centrifugation volumes in this equipment were relatively low (~ 250 mL), and thus many cycles were required, mainly in S-III. This resulted in the dilution of the biomilking product with mineral media during biomass resuspension in water. During S-IV, the use of the Thermoscientific™ Lynx4000 centrifuge allowed the centrifugation of higher volumes of cultivation broth (~750 mL), facilitating the recovery of the product solution. This better centrifugation control also avoided the concomitant dilution of the culture broth, thereby preventing higher losses in salinity levels. Despite these variations in product solution volumes and the resulting *Ect* and *Hy* concentrations, higher mass productivities were achieved at higher volumes of biomilked culture broth, ranging from approximately 71.4 to 312.8 mg-*Ect* recovered per cycle. Furthermore, the overall productivity of *Ect* + *Hy* after biomilking ranged from 7.8 to 27.2 g per kg of inlet CH_4 per day supporting the profitability of the process for biogas valorisation according to previous

Table 2
Product volume, mass of osmolytes (mg/cycle) and extraction efficiencies (EE, %) per cycle of biomilking.

Stage	Fraction of culture broth biomilked (%)	Product volume (L/cycle)	Ectoine (*Ect*)		Hydroxyectoine (*Hy*)		Y_{Ect+Hy} (g -*Ect* + *Hy* kg CH_4^{-1} d^{-1})
			mg/cycle	EE (%)	mg/cycle	EE (%)	
II	10	0.3 ± 0.0	71.4 ± 15.7	62.0 ± 14.9	10.3 ± 2.9	50.1 ± 15.7	7.8 ± 0.9
III	50	1.2 ± 0.1	196.3 ± 54.6	52.7 ± 15.6	23.8 ± 8.9	50.1 ± 17.0	18.0 ± 4.4
IV	30	0.8 ± 0.1	174.9 ± 27.2	71.2 ± 10.6	14.7 ± 4.4	82.4 ± 26.0	15.9 ± 2.7
V	60	1.4 ± 0.1	305.1 ± 18.1	62.4 ± 7.8	17.3 ± 5.1	78.3 ± 14.2	27.0 ± 2.4
VI	60 (NaCl)	1.3 ± 0.1	312.8 ± 46.7	63.7 ± 2.8	18.2 ± 7.1	72.8 ± 12.2	27.2 ± 3.9

economic analyses (Pérez et al., 2021).

3.3. Characterisation of culture and biomilking broths

Fig. 3 shows the primary physicochemical characteristics of the culture broth and the product solution obtained after biomilking. The culture broth was characterised by a relatively stable average pH of 8.4 ± 0.1 (Fig. 3), and soluble concentrations of IC, TOC and TN of 719.4 ± 58.1 mg L^{-1}, 84.3 ± 62.2 mg L^{-1}, and 167.3 ± 58.2 mg L^{-1}, respectively. The prevailing TOC concentrations were likely due to the production of metabolites from CH_4 oxidation and to soluble cell debris generated by the high shear stress imposed by the hydrodynamics in the TFB (Herrero et al., 2025), and by the centrifugation cycles during biomilking. On the other hand, the high concentrations of TN in the liquid media guaranteed an effective synthesis of both *Ect* and *Hy*. The conductivity in the culture broth decreased over time from 85.5 ± 1.2 mS cm^{-1} at the beginning of the experiment to 72.9 ± 4.3 mS cm^{-1}, which supported the need for NaCl addition during biomilking in S-VI to restore the conductivity (886 ± 1.5 mS cm^{-1}). The addition of salt during S-VI mediated the recovery of intracellular *Ect* accumulation.

Significant variations were observed in the pH of the biomilking solution, which ranged from 8.1 to 8.6. These variations in pH were primarily attributed to the composition of water. Additionally, salinity losses during biomilking resulted in a consistent decrease in the conductivity in the biomilking solution from 32.7 ± 0 mS cm^{-1} in S-II to 15.4 ± 5.3 mS cm^{-1} in S-VI (Fig. 3). Despite variations among stages, the biomilking solution contained lower concentrations of IC (213 ± 5 mg L^{-1}) and TN (107 ± 10 mg L^{-1}) but higher concentrations of TOC (273 ± 50 mg L^{-1}) than the culture broth. Considering the *Ect* and *Hy* molecular formulas ($C_6H_{10}N_2O_2$ and $C_6H_{10}N_2O_3$) and the concentrations of these osmolytes recorded in the biomilking product, shares of TOC between 35 % (S-III) and 62 % (S-II), and of TN from 29 % (S-III) to 63 % (S-V) can be attributed to the *Ect* and *Hy* content.

3.4. Bacterial diversity

After data quality control (Merging, filtration and Chimeras removal), the number of reads obtained ranged between 47,764 and 52,696 (see Supplementary Material). The Shannon diversity index values (see Supplementary Material) at the species level showed a trend to increasing diversity from S-I (2.865) to S-V (4.197) with relatively high and stable values later in S-VI (3.934) and S-VII (3.742) (see Supplementary Material).

At the phylum level, the NGS analyses revealed a high dominance of Proteobacteria (42 %-69 %) throughout the operational period (Fig. 4a). Among proteobacteria, the genera with higher dominance included *Methylomicrobium, Paracoccus, Yoonia,* and *Methylophaga*. Other phyla with significant shares were Bacteroidia, with the genera *Unidentified_Saprospiraceae and Wandonia*, which decreased from 51 % and 54 % in S-I and S-II to 7 % in S-VII; and Planctomycetes, with increasing dominances (9–24 %) of *SM1A02* from S-IV to S-VI. *Wenzhouxiangella, Lacimicrobium* and *Nitratireductor* (Proteobacteria) completed the top-10 dominant genera, which accumulated about 53 % (S-IV) to 92 % (S-II) of dominance throughout all stages. Among these dominant genera, *Methylomicrobium*, *Methylophaga* and *Paracoccus* were previously identified as ectoine producers (Reshetnikov et al., 2006; Mustakhimov et al., 2012; Cantera et al., 2020; Zhang et al., 2023). The presence of these ectoine-accumulating organisms was high throughout the experiment, with abundances of 31 % during S-I and S-II, which decreased to 16 %, 13 %, and slightly increased to 21 % during S-III, S-IV, and S-V, respectively, and increased again to 44 % and 39 % in S-VI and S-VII. Furthermore, *Methylomicrobium* and *Lacimicrobium* were positively correlated with *Ect* content, while *Paracoccus* with *Hy* content (see Supplementary Material). *Lacimicrobium,* a facultatively aerobic bacterium capable of organic carbon degradation (Zhong et al., 2016), was previously detected in a mixed inoculum for *Ect* production from biogas (Rodero et al., 2022), while *Paracoccus* was previously associated not only with *Ect* but also with *Hy* production (Severin et al., 1992).

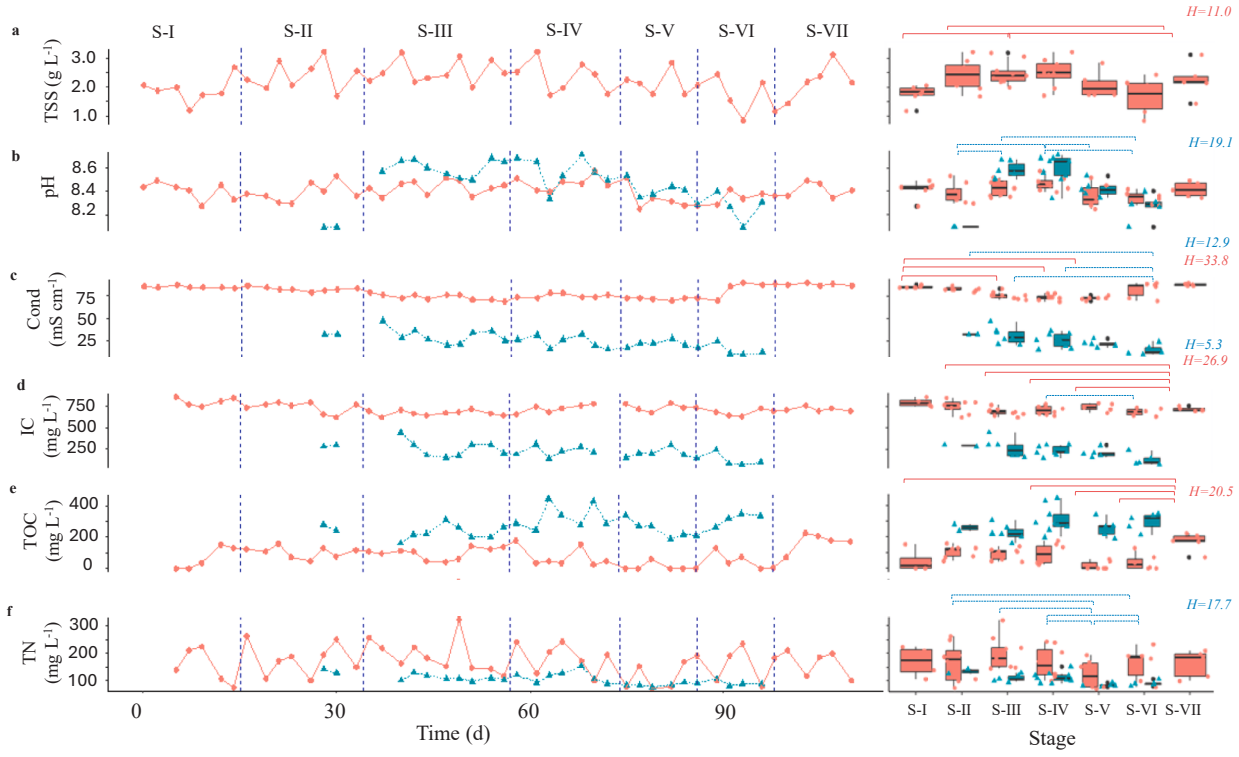


Fig. 3. Time course and boxplots of the concentrations of (a) Total suspended solids (TSS, mg L^{-1}); (b) pH; (c) Conductivity (mS cm^{-1}); (d) Inorganic Carbon (IC, mg L^{-1}); (e) Total organic carbon (TOC, mg L^{-1}); and (f) Total Nitrogen (TN, mg L^{-1}) in the culture broth (●) and in the biomilking product solution (▲). Breaks in the boxplot figures indicate the most relevant significant pairwise comparisons in Dunn's test.

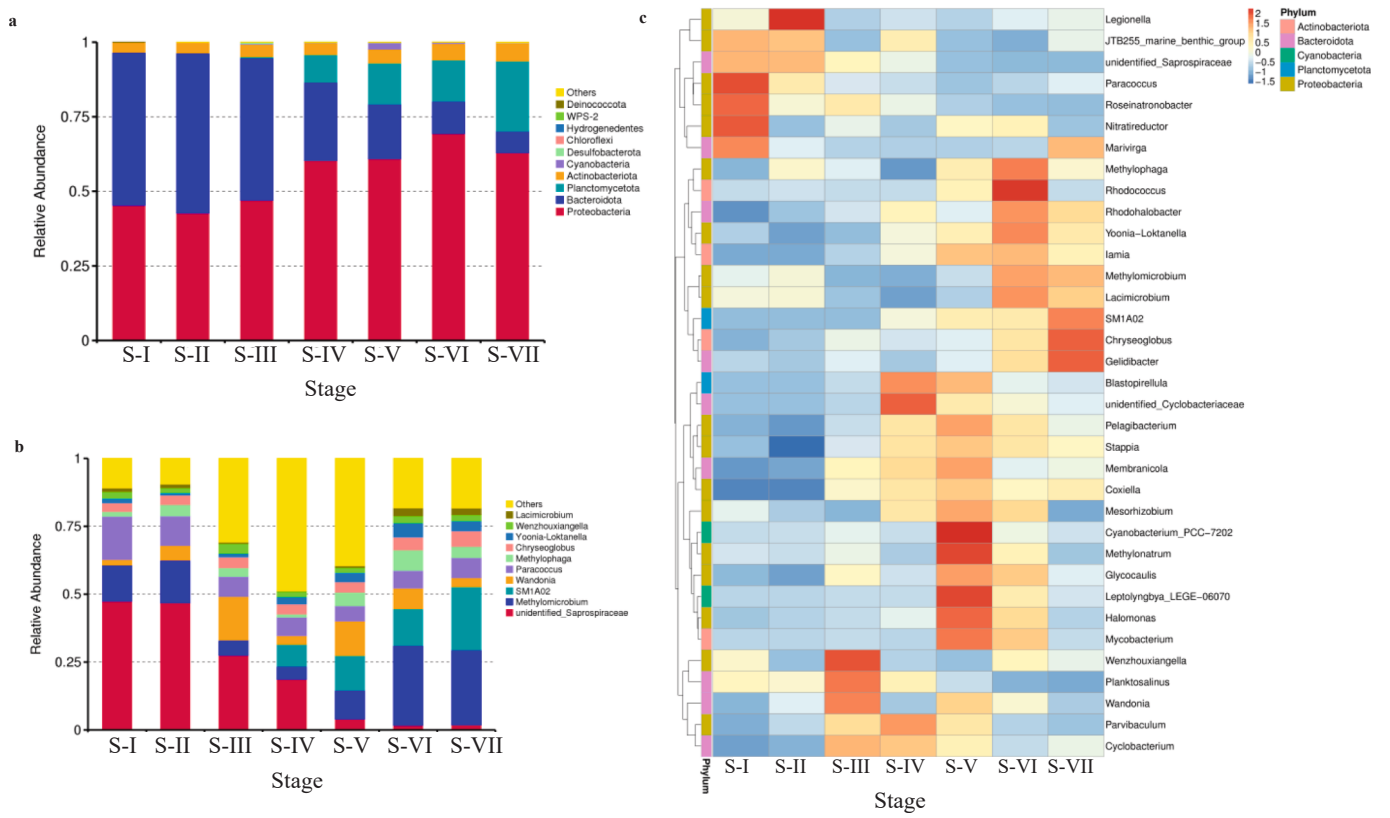


Fig. 4. Bacterial relative dominances at (a) phylum and (b) genus level as a function of the operational stage (c) Taxonomic abundance cluster heatmap (genus level).

The shifts in taxonomic dominance (Fig. 4b, 4c), including ectoine producers, indicated a high impact of biomilking on the microbial communities and the metabolic activity prevailing in the TFB. During S-I to S-III, more than 50 % of the dominance comprised *Unidentified_Saprospiraceae, Methylomicrobium*, and *Methylophaga,* shifting from S-III to S-V, accompanied by a high diversity of taxa and concomitant with lower shares of ectoine producers, mainly *Methylomicrobium*. During S-VI and S-VII, a higher presence of *Wandonia* and *SM1A02*, occurred along with the recovery of shares of ectoine producers.

The salt losses recorded during S-II to S-V were likely the main drivers of the observed changes in bacterial community composition, favouring a higher bacterial diversity (Fig. 4b and 4c). However, this increase was accompanied by a concomitant loss of ectoine accumulators and decreases in CH_4-RE, CH_4-BC and osmolytes intracellular contents. The losses in salt levels affected mainly *Methylomicrobium*, as verified by the positive correlation (see Supplementary Material) between the culture broths conductivity and *Methylomicrobium* dominance. Furthermore, a possible shortage of metabolites such as methanol, formaldehyde, or formate, typically produced during CH_4 oxidation by *Methylomicrobium* (Akberdin et al., 2018), could indirectly affect *Methylophaga* and *Paraccocus*, which likely grew on these compounds produced during methane oxidation (Kröber & Schäfer, 2019; Parekh and Spiro, 2025). The low TOC concentrations in the culture broth, recorded mainly in S-V, seemed to support this low availability of metabolites.

The restoration of salinity levels in S-VI and the cessation of biomilking operation in S-VII mediated a rapid increase in the abundances of *Methylomicrobium* to the levels recorded in S-I and S-II, supporting *Methylophaga* and *Paracoccus* activity. *Unidentified_Saprospiraceae* and *Yoonia,* two of the dominant genera, are potential denitrifiers (Kondrotaite et al., 2022; Zhao et al., 2024), while *SM1A02* is a nitrate oxidiser (Vico et al. 2021) and *Wandonia* is an aerobic halotolerant heterotroph (Lee et al., 2010). Particularly in S-IV and S-V, these genera, along with ectoine accumulators, represented only 45 % and 54 % of the diversity, suggesting that the probable shortage of metabolites hampered their activity. However, this condition allowed several other taxa (mainly aerobic heterotrophs belonging to Proteobacteria, (*Wenzhouxiangella*, *Lacimicrobium*, and *Nitratireductor*), Bacteroidota (*Cyclobacteriaceae*, *Planktosalinus* and Planctomycetota (*Blastopilelulla*)) to accumulate to about 15 % of total abundance during these stages (Fig. 4b and 4c). This elevated richness of heterotrophs may be relevant for metabolising organic byproducts of methane oxidation, enhancing methanotrophic activity through complex interactions that remain unclear (Ruiz-Ruiz et al., 2025). Overall, a very specialised halophilic or halotolerant bacterial consortium, driven by *Methylomicrobium* activity and the heterotrophic utilisation of carbon metabolites by aerobic bacteria and denitrifiers, supported methane bioconversion into *Ect* and *Hy*.

3.5. *Influence of NaCl supplementation and enhancement opportunities*

As described above, maintaining a salt concentration of 6 % in the culture broth in S-VI guaranteed process stability by recovering *Methylomicrobium* abundances, which likely enhanced other *Ect* and *Hy* producers such as *Methylophaga*, *Paracoccus* and *Lacimicrobium*. These results are in agreement with previous studies in which a consortium of haloalkaliphilic methanotrophs, methylotrophs and heterotrophs, such as *Methylophaga* or *Methylomicrobium,* enhanced ectoine production from biogas (Cantera et al., 2017b). The increase in the intracellular content of both ectoines associated with NaCl supplementation resulted in a slightly higher release of these osmolytes in the biomilking product solution, achieving average concentrations of 234.6 ± 19.3 mg-*Ect* L^{-1} and 15.4 ± 5.3 mg-*Hy* L^{-1} (Table 2), values comparable to those achieved by Cantera et al. (2017b) or Rodero et al. (2023), using residual gases and mixed inocula. Product concentrations corresponded to a production yield of 28.4 mg-*Ect* $gTSS^{-1}$, still a low value compared to yields ranging from 140 to 540 mg *Ect* $gVSS^{-1}$ in batch fermentations with pure strains (Chen et al., 2024; Ng et al., 2023) and high-titre ectoine production above 100 g L^{-1} (Lei et al., 2025). However, the

lower cost of biogas (€114 t^{-1}) compared to that of glucose (€1590 t^{-1}) and an overall production cost that may range over €214 kg^{-1} suggest a high profitability of the process (Pérez et al., 2021; El Ibrahimi et al., 2025).

In this context, long-term biomilking with salinity control can support profitable osmolyte productivities, confirming the feasibility of coupling the process to anaerobic digestion for valorising residual CH_4 and under continuous recirculation of the biomilked culture to the upstream methanotrophic cultivation. Further optimisation of the centrifugation steps and water-to-culture broth ratios during biomilking can increase extraction efficiencies. In this sense, higher water volumes such as those used by Rodero et al. (2023), who applied a higher water-to-cultivation broth ratio (1.5:4), or higher agitation times (e.g., 20 mins in Van-Thuoc et al., 2010) could result in a higher hypoosmotic shock and higher osmolyte extraction efficiencies. Indeed, the nominal increase in extraction efficiency observed during S-VI with NaCl addition also supports the hypothesis that the higher the differential in salt concentrations, the higher the quantities of osmolytes released.

4. Conclusions

Long-term biomilking operation resulted in a decrease in culture broth salinity and loss of ectoine-producers, mainly *Methylomicrobium*. Salt losses were primarily associated with biomass washing during the hypoosmotic shock. The changes in the methanotrophic consortium resulted in minimum CH_4-BE and CH_4-BC of 33.6 % and 22.1 g-CH_4 m^{-3} h^{-1}, respectively and a minimum *Ect* content of 27.9 mg $gTSS^{-1}$ in the culture broth. Restoring salt levels enabled recovery of *Methylomicrobium* dominance and *Ect* intracellular accumulation capacity, yielding up to 27.2 g-*Ect* + *Hy* kg CH_4^{-1} d^{-1} in the biomilking product and *Ect* content of 53.8 mg $gTSS^{-1}$. Despite achieving lower accumulation capacities than the 500 mg *Ect* $gVSS^{-1}$ and titers above 100 g-*Ect* L^{-1} achieved with genetically engineered organisms cultivated on expensive substrates (Lei et al., 2025), these results support the feasibility of this platform technology for commercial biogas conversion.

CRediT authorship contribution statement

Andrés Felipe Torres-Franco: Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Raquel Herrero-Lobo:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Raquel Lebrero:** Writing – review & editing, Writing – original draft, Resources, Conceptualization. **Raúl Muñoz:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Conceptualization.

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.

Acknowledgments

This investigation was financed by the EU Horizon CHEERS project, funded by the European Union's Horizon Europe Research and Innovation programme under Grant Agreement No 101060814. UK participants in Horizon Europe Project CHEERS are supported by UKRI grant numbers 10050977 Earthwatch Europe. The support of the Department of Education of the Regional Government of Castilla y León (Spain) and the European Union through the European Regional Development Fund (ERDF) (Reference: CLU-2025-2-06) is also acknowledged. The Regional Government of Castilla y León (Spain) is also acknowledged for the predoctoral contract of Raquel Herrero Lobo. Beatriz Estíbaliz Muñoz-González and Enrique José Marcos-Montero are thanked for their valuable technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2026.133920.

Data availability

Data will be made available on request.

References

Akberdin, I.R., Thompson, M., Hamilton, R., Desai, N., Alexander, D., Henard, C.A., Kalyuzhnaya, M.G., 2018. Methane utilization in *Methylomicrobium alcaliphilum* 20ZR: a systems approach. Sci. Rep. 8 (1), 2512.

Bursy, J., Pierik, A.J., Pica, N., Bremer, E., 2007. Osmotically induced synthesis of the compatible solute hydroxyectoine is mediated by an evolutionarily conserved ectoine hydroxylase. J. Biol. Chem. 282 (43), 31147–31155.

Cantera, S., Lebrero, R., Rodríguez, S., García-Encina, P.A., Muñoz, R., 2017. Ectoine biomilking in methanotrophs: a step further towards methane-based bio-refineries into high added-value products. Chem. Eng. J. 328, 44–48.

Cantera, S., Lebrero, R., Rodríguez, E., García-Encina, P.A., Muñoz, R., 2017b. 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.

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.

Chen, R., Zhu, L., Lv, L., Yao, S., Li, B., Qian, J., 2017. Optimization of the extraction and purification of the compatible solute ectoine from *Halomonas elongate* in the laboratory experiment of a commercial production project. World J. Microbiol. Biotechnol. 33, 1–7.

Chen, J., Qiao, D., Yuan, T., Feng, Y., Zhang, P., Wang, X., Zhang, L., 2024. Biotechnological production of ectoine: current status and prospects. Folia Microbiol. 69 (2), 247–258.

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. Manag. 298, 113462.

Cattaneo, C.R., Rodríguez, Y., Rene, E.R., García-Depraect, O., Muñoz, R., 2022. Biogas bioconversion into poly (3-hydroxybutyrate) by a mixed microbial culture in a novel Taylor flow bioreactor. Waste Manag. 150, 364–372.

El Ibrahimi, M., Khan, M., Ruiz-Ruiz, P., De Vrieze, J., Van Dael, M., 2025. Toward complete and sustainable biogas valorization: Ectoine synthesis using a methanotroph-microalgae consortium. J. Environ. Chem. Eng. 120093.

Fermoso, F.G., Serrano, A., Alonso-Fariñas, B., Fernández-Bolaños, J., Borja, R., Rodríguez-Gutiérrez, G., 2018. Valuable compound extraction, anaerobic digestion, and composting: a leading biorefinery approach for agricultural wastes. J. Agric. Food Chem. 66 (32), 8451–8468.

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.

He, Y.Z., Gong, J., Yu, H.Y., Tao, Y., Zhang, S., Dong, Z.Y., 2015. High production of ectoine from aspartate and glycerol by use of whole-cell biocatalysis in recombinant *Escherichia coli*. Microb. Cell Fact. 14 (1), 55.

Herrero-Lobo, R., Torres-Franco, A.F., Llamas-Ramos, W.M., Monsalvo, V., Zamora, P., Rogalla, F., Muñoz, R., 2024. Influence of design and operational parameters of a Taylor flow reactor on the bioconversion of methane to ectoines. J. Environ. Chem. Eng. 12 (6), 114323.

Herrero-Lobo, R., Torres-Franco, A.F., Lebrero, R., del Rosario Rodero, M., Muñoz, R., 2025. Evaluation of the influence of the gas residence time and biomass concentration on methane bioconversion to ectoines in a novel Taylor flow bioreactor. J. Environ. Manag. 373, 123592.

Huang-Lin, E., Tamarit, D., Lebrero, R., Cantera, S., 2024. *Guyparkeria halophila*: Novel cell platform for the efficient valorization of carbon dioxide and thiosulfate into ectoine. Bioresour. Technol. 408, 131152.

Kondrotaite, Z., Valk, L.C., Petriglieri, F., Singleton, C., Nierychlo, M., Dueholm, M.K., Nielsen, P.H., 2022. Diversity and ecophysiology of the genus OLB8 and other abundant uncultured *Saprospiraceae* genera in global wastewater treatment systems. Front. Microbiol. 13, 917553.

Kraakman, N.J., González-Martín, J., Pérez, C., Rodríguez, E., Lebrero, R., Deshusses, M. A., Munoz, R., 2023. Hydrophobic air pollutants removal at one second gas contact in a multi-channel capillary bioreactor. J. Environ. Chem. Eng. 11 (5), 110502.

Kröber, E., Schäfer, H., 2019. Identification of proteins and genes expressed by *Methylophaga thiooxydans* during growth on dimethylsulfide and their presence in other members of the genus. Front. Microbiol. 10, 1132.

Lee, D.H., Choi, E.K., Moon, S.R., Ahn, S., Lee, Y.S., Jung, J.S., Kahng, H.Y., 2010. *Wandonia haliotis* gen. nov., sp. nov., a marine bacterium of the family *Cryomorphaceae*, phylum *Bacteroidetes*. Int. J. Syst. Evol. Microbiol. 60 (3), 510–514.

Lee, Y.S., Chai, H., Cho, S., Na, J.G., Lee, J., 2022. Improved ectoine production from methane by optimization of the bio-milking process in engineered *Methylomicrobium alcaliphilum* 20Z. Korean Chem. Eng. Res. 60 (3), 392–397.

Lei, Z., Chen, X., Yuan, L., Wu, J., Yao, J., 2025. Metabolic control for high-efficiency ectoine synthesis in engineered *Escherichia coli*. ACS Synth. Biol. 14 (8), 3177–3185.
Louis, P., Galinski, E.A., 1997. Characterization of genes for the biosynthesis of the compatible solute ectoine from *Marinococcus halophilus* and osmoregulated expression in *Escherichia coli*. Microbiol 143 (4), 1141–1149.
Marcos-Rodrigo, E., Lebrero, R., Muñoz, R., Sousa, D.Z., Cantera, S., 2025. Syngas biological transformation into hydroxyectoine. Bioresour. Technol. 417, 131842.
Mustakhimov, I.I., Reshetnikov, A.S., Fedorov, D.N., Khmelenina, V.N., Trotsenko, Y., 2012. Role of EctR as transcriptional regulator of ectoine biosynthesis genes in *Methylophaga thalassica*. Biochemistry (Mosc.) 77, 857–863.
Ng, H.S., Wan, P.K., Kondo, A., Chang, J.S., Lan, J.C.W., 2023. Production and recovery of ectoine: a review of current state and future prospects. Processes 11 (2), 339.
Orhan, F., Ceyran, E., 2023. Identification of novel halophilic/halotolerant bacterial species producing compatible solutes. Int. Microbiol. 26 (2), 219–229.
Parekh, T., Spiro, S., 2025. Regulation of methylotrophy in *Paracoccus denitrificans*. Adv. Microb. Physiol. 87, 163–208.
Pastor, J.M., Salvador, M., Argandoña, M., Bernal, V., Reina-Bueno, M., Csonka, L.N., Cánovas, M., 2010. Ectoines in cell stress protection: uses and biotechnological production. Biotechnol. Adv. 28 (6), 782–801.
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 Sustain. Chem. Eng. 9 (51), 17371–17380.
Reshetnikov, A.S., Khmelenina, V.N., Trotsenko, Y.A., 2006. Characterization of the ectoine biosynthesis genes of haloalkalotolerant obligate methanotroph "*Methylomicrobium alcaliphilum* 20Z". Arch. Microbiol. 184, 286–297.
Rodero, M., Herrero-Lobo, R., Pérez, V., Muñoz, R., 2022. Influence of operational conditions on the performance of biogas bioconversion into ectoines in pilot bubble column bioreactors. Bioresour. Technol. 358, 127398.
Rodero, M.R., Carmona-Martínez, A.A., Martínez-Fraile, C., Herrero-Lobo, R., Rodríguez, E., García-Encina, P.A., Muñoz, R., 2023. Ectoines production from biogas in pilot bubble column bioreactors and their subsequent extraction via bio-milking. Water Res. 245, 120665.
Rodero, M., Pérez, V., Muñoz, R., 2024. Optimization of methane gas-liquid mass transfer during biogas-based ectoine production in bubble column bioreactors. J. Environ. Manag. 366, 121811.
Ruiz-Ruiz, P., Mohedano-Caballero, P., De Vrieze, J., 2025. Ectoine production through a marine methanotroph-microalgae culture allows complete biogas valorization. J. Environ. Manag. 375, 124223.
Sauer, T., Galinski, E.A., 1998. Bacterial milking: a novel bioprocess for production of compatible solutes. Biotechnol. Bioeng. 57 (3), 306–313.
Severin, J., Wohlfarth, A., Galinski, E.A., 1992. The predominant role of recently discovered tetrahydropyrimidines for the osmoadaptation of halophilic eubacteria. Microbiol 138 (8), 1629–1638.
Singh, R., Ryu, J., Kim, S.W., 2019. Microbial consortia including methanotrophs: some benefits of living together. J. Microbiol. 57 (11), 939–952.
Tanimura, K., Matsumoto, T., Nakayama, H., Tanaka, T., Kondo, A., 2016. Improvement of ectoine productivity by using sugar transporter-overexpressing *Halomonas elongata*. Enzyme Microb. Technol. 89, 63–68.
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 (1), 46–51.
Vico, P., Iriarte, A., Bonilla, S., Piccini, C., 2021. Metagenomic analysis of *Raphidiopsisraciborskii* microbiome: beyond the individual. Biodivers. Data J. 9, e72514.
Zhang, K., Zeng, Q., Jiang, R., Shi, S., Yang, J., Long, L., Tian, X., 2023. Three novel marine species of *Paracoccus*, *P. aerodenitrificans* sp. nov., *P. sediminicola* sp. nov. and *P. albus* sp. nov., and the characterization of their capability to perform heterotrophic nitrification and aerobic denitrification. Microorganisms, 11(6), 1532.
Zhao, Z., Zhao, Y., Marotta, F., Xamxidin, M., Li, H., Xu, J., Wu, M., 2024. The microbial community structure and nitrogen cycle of high-altitude pristine saline lakes on the Qinghai-Tibetan plateau. Front. Microbiol. 15, 1424368.
Zhong, Z.P., Liu, Y., Wang, F., Zhou, Y.G., Liu, H.C., Liu, Z.P., 2016. *Lacimicrobium alkaliphilum* gen. nov., sp. nov., a member of the family *Alteromonadaceae* isolated from a salt lake. Int. J. Syst. Evol. Microbiol. 66 (1), 422–429.