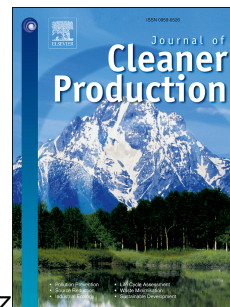# Accepted Manuscript

Continuous abatement of methane coupled with ectoine production by *Methylomicrobium alcaliphilum 20Z* in stirred tank reactors: A step further towards greenhouse gas biorefineries

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Please cite this article as: Cantera S, Lebrero R, Rodríguez E, García-Encina PA, Muñoz R, Continuous abatement of methane coupled with ectoine production by *Methylomicrobium alcaliphilum 20Z* in stirred tank reactors: A step further towards greenhouse gas biorefineries, *Journal of Cleaner Production* (2017), doi: 10.1016/j.jclepro.2017.03.123.
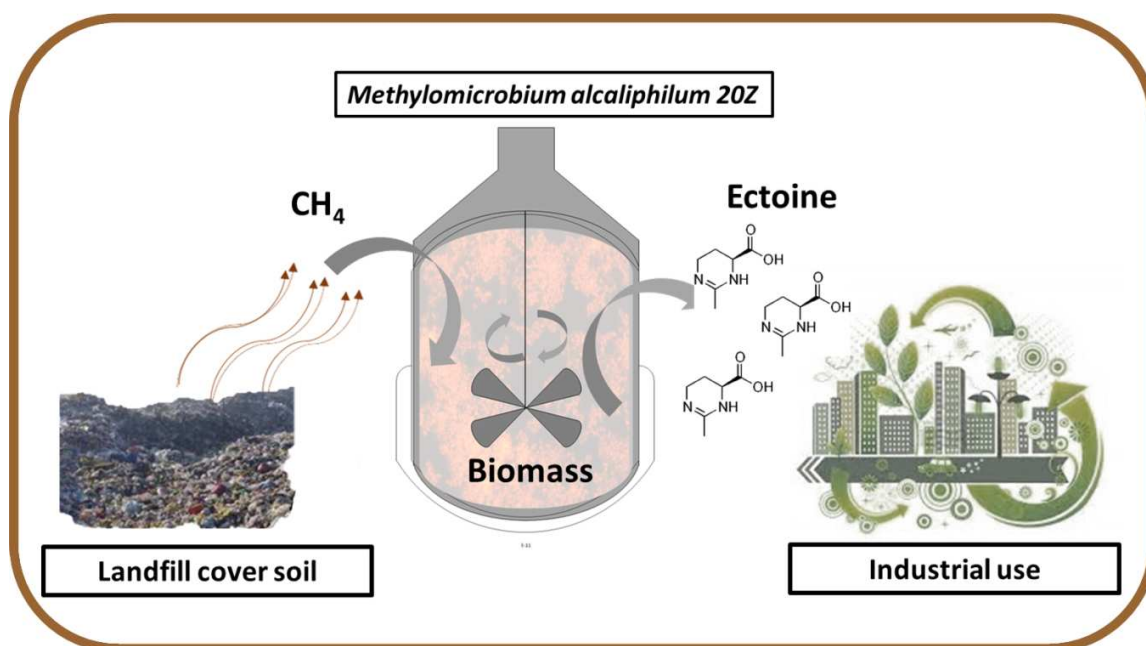
This research investigated for the first time the valorization in continuous stirred tank reactors of dilute CH_4 emissions into ectoine, a microbial molecule with a high retail value. The results here obtained supported the technical viability of a new generation of GHG biorefineries and revealed that high intra-cellular ectoine yields were obtained using methane and high Cu^{2+} concentration promoted the excretion to the cultivation broth of 20 % of the total ectoine synthesized during continuous operation.

6456 words

Continuous abatement of methane coupled with ectoine production by *Methylomicrobium alcaliphilum 20Z* in stirred tank reactors: a step further towards greenhouse gas biorefineries

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Abstract:

This study demonstrates for the first time the feasibility of producing ectoine (a high added value osmoprotectant intensively used in the cosmetic industry) during the continuous abatement of diluted emissions of methane by *Methylomicrobium alcaliphilum 20Z* in stirred tank reactors under non-sterile conditions. An increase in NaCl concentration in the cultivation broth from 3 to 6 % increased the intra-cellular ectoine yield by a factor of 2 (from 16.5 to 37.4 mg ectoine (g biomass)$^{-1}$), while high stirring rates (600 rpm) entailed a detrimental cellular stress and 3 times lower ectoine yields (5.6 mg ectoine (g biomass)$^{-1}$) compared to process operation at 300 rpm. An increase in Cu^{2+} concentration from 0.05 to 25 µM enhanced methane abatement by a factor of 2 (up to elimination capacities of 24.5 g m^{-3} h^{-1}), did not enhance intra-cellular ectoine production but promoted the excretion to the cultivation broth of 20 % of the total ectoine synthesized regardless of the NaCl concentration and stirring rate. The results obtained by culture hybridization with the specific probe Mγ1004 showed that *Methylomicrobium alcaliphilum 20Z* accounted for more than 80 % of the total bacterial population in most experimental runs. This work confirmed the technical feasibility of a new generation of biorefineries based on the abatement of diluted CH_4 emissions using extremophile methanotrophs.

Keywords: Ectoine, climate change, methane abatement, methane biorefinery, *Methylomicrobium*

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1. Introduction

Methane (CH_4), with a global warming potential 25 times higher than that of CO_2 in a 100 y horizon, is nowadays the second most relevant greenhouse gas (GHG) emitted to the atmosphere (European Environmental Agency, 2015). CH_4 can be used as an energy vector for the production of electricity and heat when its concentration is higher than 20 %, however, more than 56 % of the anthropogenic CH_4 emissions worldwide contain concentrations of this GHG lower than 5 % (Estrada et al., 2014; IPCC, 2014; EEA, 2015). CH_4 abatement using conventional physical/chemical technologies is either inefficient or too costly at such low concentrations, and often entails a large CO_2 footprint (Estrada et al., 2014; Nikiema et al., 2007). In this context, biological treatment technologies can become a low cost and environmentally friendly alternative to their physical/chemical counterparts for the treatment of diluted CH_4 emissions (López et al., 2013).

However, the widespread implementation of methane treatment biotechnologies is still restricted by i) the low mass transfer of CH_4 from the emission to the bacterial community due to its high hydrophobicity, which entails high investment and operating costs; and ii) the lack of knowledge about the potential industrial applications of methanotrophic bacteria for the bioconversion of CH_4 into high-added value products, which would significantly enhance the economic viability of the process (López et al., 2013; Strong et al., 2016). Indeed, the biological oxidation of diluted CH_4 emissions combined with the production of high added value products could be, if properly tailored, a cost-competitive approach to mitigate climate change.

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is one of the most valuable bioproducts synthesised by microorganisms, retailing in the pharmaceutical industry at approximately US$1000 kg^{-1} (among the most important pharmaceutical companies that produce ectoine nowadays can be found Merk, Johnson & Johnson, Larens, Bioderma…). The global

demand of this compound accounts for 15000 tones year^{-1} (Strong et al., 2016). This inmino acid, which is synthesized by bacteria to survive in salt-rich environments, is an effective stabilizer for enzymes, DNA-protein complexes and nucleic acids (Pastor et al., 2010). Currently, ectoine is commercially produced using the halophilic heterotroph *Halomonas elongata*. The most common industrial process, namely *bio-milking,* consists of a fed-batch two-stage at different salt concentrations (12 and 0%) to obtain first a high density culture (25 g L^{-1}) that is subsequently subjected to a hypoosmotic shock. The sudden decrease in medium salinity results in the excretion of ectoine from the cell to the culture broth, where the product is collected for its downstream purification. Despite the extensive design and operation experience in this industrial process, the high cost of the glucose required as a carbon source, and the need for an intensive O_2 supply, entail high operating costs (Pastor et al., 2010; Strong et al., 2015).

In 1999, Khmelenina et al. (1999) demonstrated that moderately halotolerant methanotrophs, such as *Methylomicrobium alcaliphilum 20Z*, could express the three specific enzymes involved in ectoine synthesis, and were thus able to accumulate ectoine inside the cell (Kaluzhnaya et al., 2001; Khmelenina et al., 1999; Khmelenina et al., 2000; Reshetnikov et al. 2006). These studies, conducted at high CH_4 concentrations, represented the first proof of the ability of CH_4-oxidizing bacteria to produce ectoine (Khemelenina et al., 2000; But et al., 2013, Strong et al., 2016). Further studies demonstrated that the bio-conversion of CH_4 into ectoine by the methanotrophic ectoine-producing strain *Methylomicrobium alcaliphilum 20 Z* could be carried out batch wise. Moreover, it was observed that environmental conditions involving CH_4 (2-20 %), Cu^{2+} (0.05-50µM) and NaCl (0-9 %) concentrations, as well as temperature (25-35 ºC), were crucial to boost ectoine synthesis and the specific CH_4 biodegradation rate (Cantera et al., 2016b). This finding supports the treatment of diluted CH_4 emissions coupled with the synthesis of ectoine in suspended growth bioreactors which would potentially reduce the costs associated to ectoine

production while boosting climate change mitigation via active CH_4 abatement. In this context, the optimization of the cultivation conditions to promote ectoine accumulation and its continuous excretion from the cell to the culture broth, either following a process similar to the currently used with *Halomonas elongata*, namely "bio-milking", or by promoting the natural excretion of ectoine to the culture broth in bioreactors, is crucial to ensure the technical and economic viability of the process (Cantera et al., 2016b).

The present study aimed at systematically elucidating the influence of copper (Cu^{2+}), NaCl concentrations and stirring rate on the continuous abatement of methane combined with the production of extra and intra-cellular ectoine in stirred tank bioreactors using the strain *M. alcaliphilum 20Z*.

2. Materials and Methods

2.1. Chemicals and mineral salt medium

A high-alkalinity mineral salt medium (MSM) with a final pH of 9.0 was used (Kalyuzhnaya et al. 2008). NaCl and $CuCl_2 \cdot 2H_2O$ were added to the MSM at the concentrations tested in each experimental run (Table 1). Unless otherwise specified, all chemicals and reagents were obtained from Panreac (Barcelona, Spain) with a purity higher than 99.0 %. CH_4 (> 99.5 %) was purchased from Abello-Linde S.A (Barcelona, Spain).

2.2. Microorganisms and inoculum preparation

Methylomicrobium alcaliphilum 20Z, an halophilic alkalitolerant methanotrophic strain able to synthesize ectoine (Kalyuzhnaya et al., 2008), was acquired from DSMZ (Leibniz-Institut, Germany). An aliquot of 1 mL of *M. alcaliphilum 20Z* stock liquid culture was inoculated in 120 mL glass bottles containing 90 mL of 3 % NaCl/0.05 µM Cu^{2+} MSM. The bottles were closed with gas-tight butyl septa and aluminum caps, and CH_4 was then injected to the headspace in

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order to reach an initial concentration of 50 % v/v air/CH$_4$. The inocula were incubated at 25 ℃ under orbital agitation at 220 rpm to a final biomass concentration of 0.1 ± 0.06 g L^{-1}.

2.3 Experimental set-up and operational conditions

1-L jacketed stirred tank reactors (STR) (Afora S.A., Spain) equipped with a Rushton turbine were used for the continuous abatement of CH$_4$ coupled with ectoine production. The STRs were filled with 950 mL of MSM and 50 mL of the inoculum previously described. A 0.033 L min^{-1} CH$_4$-laden air emission containing 26.8 ± 2.1 g CH$_4$ m^{-3} ($\approx 4\%$), corresponding to a methane load of 53 g m^{-3} h^{-1}, was fed to the STRs via 10 μm porous stainless steel diffusers located at the bottom of the reactors. This polluted air emission was obtained by mixing a continuous pure CH$_4$ stream (from a CH$_4$ gas cylinder stored in a safety gas cylinder cabinet and regulated by a mass flow controller, Aalborg, USA) with a continuous pre-humidified air flow, resulting in a gas empty bed residence time (EBRT) of 30 min. 500mL of culture broth were replaced by fresh MSM every two days to prevent the accumulation of inhibitory metabolites and to maintain optimal nutrient concentrations. 400 mL of the total 500 mL drawn were centrifuged at 10000 rpm for 10 min and the biomass pellet was returned to the bioreactor prior resuspension in 500 mL of fresh MSM. The remaining 100 mL of aqueous cultivation broth were used for the determination of the biomass concentration (measured as culture absorbance and total suspended solids (TSS)), total nitrogen (TN), as well as intra and extra-cellular ectoine. Samples for the determination of TSS, TN and ectoine concentrations were drawn every two days. Six different operational conditions were tested (Table 1). In experimental runs 1 and 2, the stirring rate and Cu^{2+} concentration were fixed at 300 rpm and 0.05 μM, respectively, while two salt concentrations (3 and 6 % NaCl, respectively) were used in order to test the influence of medium salinity on CH$_4$ abatement and ectoine production. The influence of NaCl concentration (3 and 6 %) was also evaluated at a Cu^{2+} concentration of 0.05 μM and a stirring rate of 600 rpm in

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experimental runs 3 and 4. Finally, experimental runs 5 and 6 were carried out at a Cu^{2+} concentration of 25 µM, 300 rpm and NaCl concentrations of 3% and 6 %, respectively, in order to assess the influence of Cu^{2+} on the simultaneous production of ectoine and CH_4 removal (by comparison with experimental runs 1 and 2). No experimental runs at a Cu^{2+} concentration of 25 µM and agitation rate of 600 rpm were performed due to the poor process performance recorded in test 3 and 4. All experimental runs were maintained for 50 days, which ensured process operation under steady state conditions. Prior to inoculation, an abiotic test with MSM was performed for 5 days at the above described operational conditions to assess any potential removal of CH_4 by adsorption or photodegradation in the experimental set-up.

<Table I>

Temperature was maintained at 25 °C in all experiments. Distilled water was weekly added to compensate water evaporation losses. Steady state conditions were achieved when the elimination capacity (CH_4-EC) and CO_2 production rates ($TPCO_2$) deviated <10 % from the mean for at least 20 days. Gas samples for CH_4 and CO_2 analysis were periodically taken from the sampling ports located at the inlet and outlet of the bioreactors using 100 µl gas-tight syringes (HAMILTON, Australia). Biomass samples were taken at the end of each steady state for the identification and quantification of *Methylomicrobium alcaliphilum 20Z* by double labeling of oligonucleotide probes fluorescence *in situ* hybridization (DOPE-FISH).

2.4 Analytical procedures

The intra-cellular ectoine contained in 2 mL of cultivation broth was extracted to the extra-cellular medium according to Cantera et al. (2016b). The specific intra-cellular ectoine concentration (g ectoine g biomass^{-1}) was calculated using the corresponding TSS concentration (g L^{-1}). An aliquot of 1 mL of cultivation broth was also drawn and filtered through 0.22 µM

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filters (Filter-lab, Barcelona) to determine the extra-cellular ectoine concentration. The concentration of ectoine was measured by HPLC-UV in a 717 plus auto-sampler (Waters, Bellefonte, USA) coupled with a UV Dual λ Absorbance detector (Waters, Bellefonte, USA) at 210 nm and 40 ℃ using a LC-18 AQ + C Supelcosil column (Waters, Bellefonte, USA) and a C18 AQ + pre-column (Waters, Bellefonte, USA). A phosphate buffer, consisting of 0.8 mM $K_2HPO_4 \cdot 3H_2O$ and 6.0 mM $Na_2HPO_4 \cdot 12H_2O$, was used as a mobile phase at 25 ℃ and a flow rate of 1 mL min^{-1} (Tanimura et al., 2013). Ectoine quantification was carried out using external standards of commercially available ectoine ((S)-b-2-methyl-1,4,5,6-tetrahydro-pyrimidine-4-carboxylic acid, purity 95 %, Sigma Aldrich, Spain). The ectoine retention time ranged from 2.32 to 2.35 min depending on the column pressure. The detection and quantification limits (DL and QL, respectively) were calculated via determination of the signal-to-noise ratio. In this sense, a signal-to-noise ratio of 3:1 - 2:1 is considered acceptable for estimating the detection limit, while a signal-to-noise ratio of 10:1 is necessary to determine the quantification limit (ICH Expert working Group, 2005). The DL and QL of ectoine in our specific MSM was 0.4 mg L^{-1} and 0.65 mg L^{-1}, respectively.

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

Culture absorbance measurements at 650 nm were conducted using a Shimadzu UV-2550 UV/Vis spectrophotometer (Shimadzu, Japan). TSS concentration was measured according to Standard Methods (American Water Works Association, 2012). pH was determined using a pH-meter Basic 20 (Crison, Spain), while the concentrations of TOC and TN were measured using a Shimadzu TOC-VCSH analyzer (Japan) equipped with a TNM-1 chemiluminescence module.

2.5. *Fluorescence in situ hybridization of the microbial cultures*

Methylomicrobium cells were identified and enumerated by Fluorescence *In Situ* Hybridization with double labeling of oligonucleotide probes (DOPE-FISH), which improves signal intensity and increases rRNA accessibility (Stoecker et al., 2010). A Cy3-double labeled oligonucleotide probe specific for *Methylomicrobium* genus (Mγ1004) (Eller et al., 2001; Stoecker et al., 2010) was used for quantification in comparison to DAPI-stained bacteria. The samples drawn at the end of each experimental run (250 μL) were fixed in 4 % (*w/v*) paraformaldehyde (750 μL) for 3 h and then washed three times with phosphate-buffered saline (PBS) medium and preserved in 96 % (*v/v*) alcohol. Aliquots of 10 µL were placed on glass microscope slides and dehydrated with ethanol at 50, 80 and 96 % (*v/v*). Hybridization was carried out at 46 °C for 12 h. The hybridization buffer contained 360 μL 5M NaCl, 40 μl 1MTris/HCl, 4μL 10 %SDS and Milli-Q water to a final volume of 2 mL. Hybridization stringency was set at 0 % formamide in the hybridization buffer as indicated in literature (Eller et al., 2001). After hybridization, the slides were submerged in 50 ml washing buffer for 20 minutes to remove unbound oligonucleotides. Total DNA was stained using DAPI (4P, 6-diamidino-2-phenylindole) (Sigma, Spain). After air-drying at room temperature, cover glasses were mounted with 2 μL Citifluor to reduce the fading of the fluorescent dyes used for labelling (Citifluor Ltd., UK). For quantitative FISH analysis, 15 images were randomly obtained from each condition using a Leica DM4000B microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DFC300FX camera. The relative bio-volumes (percentage) of *Methylomicrobium* from the total DAPI-stained bacterial population were calculated using the software DAIME (Daims et al., 2006). The averaged bio-volume fractions of *Methylomicrobium* and the standard error of the mean were calculated for each experimental run. Phase contrast images were also acquired to monitor the appearance of *Methylomicrobium* population at the end of the six experimental runs.

2.6. Data analysis

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

3. Results and Discussion

3.1 Influence of operational conditions on intra-cellular ectoine production

The concentration of intra-cellular ectoine peaked at days 5-7 regardless of the operational conditions evaluated (Figure 1). The intra-cellular ectoine yield sharply decreased afterwards, increasing again to achieve another relative maximum concentration between days 11 and 14 (Figure 1). Finally, the intra-cellular ectoine concentration decreased and stabilized from day 28 onward (Figure 1).

< Figure 1>

The correlation between an intensive production of ectoine and *M. alcaliphilum 20Z* exponential growth has been previously reported in batch wise cultivations. Khemelenina et al. (2000) observed that intra-cellular ectoine concentration peaked in the mid-exponential growth phase of *M. alcaliphilum 20Z*, and decreased afterwards during the growth-retardation phase, which suggested that ectoine can be used for the synthesis of other cell constituents. Cantera et al. (2016b) confirmed the over-production of intra-cellular ectoine during the exponential growth phase of *M. alcaliphilum 20Z*. This phenomenon could be associated to a hyperosmotic shock that initially resulted in an over-expression of the ect*abc* operon followed by a decrease in the concentration of ectoine, which was likely re-assimilated by cell metabolism.

The steady state intra-cellular ectoine yield was comparatively evaluated at the operational conditions tested (Figure 2). Cu^{2+} concentration did not show an effect on ectoine accumulation despite Cu^{2+} is a key micronutrient for type I methanotrophs (Semrau et al., 2010). Nevertheless, NaCl concentration showed a major influence on the accumulation of intra-cellular ectoine in the long-term operation. A NaCl concentration of 6 % was identified in this study as the optimum salinity for the accumulation of intra-cellular ectoine, supporting average concentrations of 31.4 ± 1.5 and 37.4 ± 3.8 mg ectoine (g biomass)$^{-1}$ at 300 rpm under low and high Cu^{2+} concentrations (experimental runs 2 and 6), respectively. On the contrary, a NaCl concentration of 3 % supported lower ectoine yields (17.6 ± 0.9 and 16.5 ± 1.8 mg ectoine (g biomass)$^{-1}$ at 300 rpm and 0.05 and 25 µM (experimental runs 1 and 5), respectively). In spite of the improved ectoine accumulation detected at higher salt concentrations (~ 2.2 times higher), this increase was lower compared with previous studies carried out batch wise, which reported ectoine productions up to 4 times higher when increasing the cultivation broth salinity from 3 to 6-7 % during the exponential growth phase (But et al., 2013; Cantera et al., 2016b). In contrast, the increase in agitation rate from 300 to 600 rpm negatively affected the accumulation of intra-cellular ectoine. In the experimental run 3 (3% NaCl and 600 rpm), the accumulation of intra-cellular ectoine decreased by a factor of 3 % (5.6 ± 2.9 mg ectoine (g biomass)$^{-1}$) compared to the experimental runs at 3% NaCl and 300 rpm. In the case of the experimental run 4 (6 % NaCl and 600 rpm) the accumulation of intra-cellular ectoine decreased by a factor of 1.5 (23.8 ± 1.1 mg ectoine (g biomass)$^{-1}$) compared to the experimental runs at 6% NaCl and 300 rpm. Although higher agitation rates can enhance the mass transfer of methane from the emission to the aqueous microbial community (Cantera et al., 2015), they can also induce a pernicious cellular stress, which resulted in a severe damage of the *Methylomicrobium alcaliphilum 20Z* culture.

<Figure 2>

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3.2. Influence of operational conditions on ectoine excretion

Extra-cellular ectoine was always detected under all operational conditions but with a different accumulation pattern to that recorded for the intra-cellular ectoine. In this context, the maximum extracellular ectoine concentration at 300 rpm and 0.05 µM Cu^{2+} was recorded by day 27 at 3 % NaCl (1.9 mg L^{-1}, corresponding to 1.3 mg g^{-1}) and by day 22 at 6 % NaCl (2.2 mg L^{-1}, corresponding to 1.5 mg g^{-1}) (experimental runs 1 and 2, respectively). However, negligible extra-cellular ectoine concentrations were detected from day 29 onward, regardless of the culture salinity (Figure 3a). At 600 rpm and 0.05 µM Cu^{2+} the maximum ectoine excreted to the medium was 3.1 mg L^{-1} (corresponding to 3.4 mg g^{-1}) at 3 % NaCl on day 13 and 3.8 mg L^{-1} (corresponding to 3.8 mg g^{-1}) at 6 % NaCl on day 13 (experimental runs 3 and 4, respectively). Ectoine excretion remained stable until the end of the operation at steady values of 1.3 ±0.2 and 1.7 ±0.4 mg L^{-1}, respectively (Figure 3b). The presence of ectoine in the extra-cellular medium along the 50 days of bioreactor operation at agitation rates of 600 rpm was likely mediated by a mechanical cell disruption induced by shear stress.

M. alcaliphilum 20Z is not described as a strain able to naturally excrete ectoine to the culture broth (Khmelenina et al., 1999; Reshetnikov et al., 2006). Therefore, the cost-efficient industrial production of ectoine by this strain requires a two-stage fed-batch process to first attain a high cell density culture with a high concentration of intra-cellular ectoine, and then excrete the accumulated ectoine by hypoosmotic shocks (Pastor et al., 2010; Strong et al., 2016). However, this study showed that at a Cu^{2+} concentration of 25 µM and 300 rpm the extra-cellular concentrations recorded were significantly higher than at 0.05 µM Cu^{2+}. Ectoine excretion remained low during the first week of operation, but increased up to 5.9 ± 0.6 and 12.9 ± 0.7 mg L^{-1} by the end of experimental runs 5 and 6, corresponding to 6.1 ± 0.9 and 8.3 ± 1.2 mg g^{-1}, respectively. Recent literature studies have reported that an increase in Cu^{2+} concentration

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decreased the expression-level of the proteins conforming the S-layer in *M. alcaliphilum 20Z* (Shchukin et al., 2011), which could support the higher secretion of ectoine at high Cu^{2+} concentrations.

<Figure 3>

Low Cu^{2+} concentrations (experimental runs 1, 2, 3 and 4) always resulted in the accumulation of intra-cellular ectoine, with only ~ 1% of that intra-cellular ectoine excreted to the culture broth. However, process operation at high Cu^{2+} concentrations (25 μM) supported similar intra-cellular ectoine concentrations, but with an ectoine excretion of ~ 22 % of the total ectoine synthesized. In this sense, the total concentration of ectoine in non-ectoine excreting strains is equal to the intra-cellular concentration required to preserve the osmotic balance, while ectoine excreting strains maintain the required concentrations of intra-cellular ectoine despite releasing ectoine into the culture broth (Lang et al., 2011). Nevertheless, our study showed that modifications in the cultivation conditions can promote the excretion of ectoine by inducing changes in cell physiology. Therefore, those conditions that promote the excretion of ectoine in non-naturally excreting strains can enhance the cost-competitiveness of industrial ectoine production since no further mechanical extraction would be needed.

<Figure 4>

3.3 Influence of operational conditions on methane abatement

A constant pH of 8.6 ± 0.4 was recorded in all experimental runs regardless of the operating conditions, which favored the growth of the alkalophilic *M. alcaliphilum 20Z* while preventing opportunistic contamination. The concentration of nitrogen was also daily monitored as total nitrogen and maintained between 100 and 130 mg L^{-1} to avoid nitrogen limitation, since nitrogen has been previously identified as a key factor for ectoine synthesis (Khmelenina et al., 2000). No

significant CH_4 degradation occurred along the abiotic removal test, as shown by the negligible difference (<1%) between inlet and outlet CH_4 gas concentrations in the STR.

Process operation at low Cu^{2+} concentrations (experimental runs 1-4) was characterized by a steady CH_4 abatement performance from day ~27 onward, while both CH_4-EC and $TPCO_2$ stabilized by day ~23 when operating at high Cu^{2+} concentrations. NaCl concentration did not influence significantly CH_4-ECs at 300 rpm and low Cu^{2+} concentrations, which remained constant at 16.5 ± 2.0 g CH_4 m^{-3} h^{-1} at 3 % NaCl and 14.8 ± 1.1 g CH_4 m^{-3} h^{-1} at 6 % NaCl (Figure 5). Methane has an inherently low solubility in water based on its hydrophobic nature. In this regard, the dimensionless CH_4 partition coefficient in water is 30 at 25 ℃ (Rocha-Ríos et al., 2009), which often entails a low CH_4 availability to the microbial community. Moreover, there is a consistent evidence that CH_4 solubility in water gets reduced at higher cultivation broth salinities, thus limiting even more the mass transfer of CH_4 from the emission to the microbial community (Duan and Mao, 2006). However, in this study no significant effect of salinity to the methane mass transfer was recorded, which always limited the process under steady state (Figure S2).

NaCl concentration did affect the growth of *M. alcaliphilum 20Z* during the initial stages of experimental run 2 (300 rpm, 0.05 Cu^{2+} and 6% NaCl), which resulted in a longer lag phase (19 days compared to 10 days at 3% NaCl). However, no significant differences between the steady state biomass concentrations were observed at low and high salt concentrations (Figure 6).

<*Figure 5*>

On the contrary, CH_4 abatement was significantly affected by the agitation rate. Hence, the CH_4-ECs recorded at 600 rpm (CH_4-ECs of 10.1 ± 1.1 g CH_4 m^{-3} h^{-1} at 3 % NaCl and 5.0 ± 1.0 g CH_4 m^{-3} h^{-1} at 6 % NaCl) were significantly lower than those achieved at 300 rpm (Figure 5). Higher agitation rates often support a better mass transfer of methane from the gas to the microbial

community, thus enhancing CH_4-EC (Estrada et al., 2014; Cantera et al. 2015). However, higher agitation rates in our study promoted an unexpected cell disruption as a result of a high shear stress on *M. alcaliphilum 20Z* (Figure S2). Indeed, the concentration of biomass (g L^{-1}) at 300 rpm was two times higher than that at 600 rpm (Figure 6), which shifted the mass transfer limitation typically encountered in methane-treating bioreactors to a microbial activity limitation. Surprisingly, despite lower CH_4-ECs were achieved at 600 rpm, the CO_2 productions recorded at 300 rpm were significantly similar to those achieved at 600 rpm. The $TPCO_2$ at 3 and 6 % were, respectively, 11.5 ± 2.3 and 11.7 ± 0.83 g CO_2 m^{-3} h^{-1} under 300 rpm, and 10.2 ± 3.0 and 10.6 ± 0.9 g CO_2 m^{-3} h^{-1} under 600 rpm. Thus, the mineralization ratios at 300 rpm were 47.0 ± 7.7 at 3% NaCl and 43.6 ± 7.7 at 6% NaCl, which entails that more than 50% of the C-CO_2 is used for biomass production. However, the average mineralization ratios recorded at 600 rpm were 79.6 ± 9.1 % at 3% NaCl and 79.9 ± 10.0 % at 6% NaCl. This is in agreement with literature studies reporting a higher share of the organic substrate being directed to energy-yielding reactions under cell stress scenarios (Chung et al., 2006).

Finally, the increase in Cu^{2+} concentration from 0.05 to 25 µM enhanced methane abatement regardless of the salinity in the cultivation broth, with CH_4-ECs of 24.5 ± 1.8 and 22.4 ± 2.1 g CH_4 m^{-3} h^{-1} at 3 and 6 % NaCl, respectively. This finding confirmed the key role of Cu^{2+} on the expression of the enzyme particulate methane monooxygenase in type I methanotrophs, and revealed that CH_4 abatement and ectoine production were limited by Cu^{2+} availability during experimental runs 1-4 (Semrau et al., 2010, Cantera et al., 2016a). Similarly to the results obtained at low Cu^{2+} concentrations, a higher salt content increased the culture lag-phase but did not influence the steady state CH_4-ECs achieved. The $TPCO_2$ of 25.4 ± 3.8 and 26.7 ± 2.8 g CO_2 m^{-3} h^{-1} recorded in experimental runs 4 and 5, respectively, yielded average mineralization ratios of 55.8 ± 6.0 % and 57.4 ± 5.0 %. No significant difference between the steady state biomass

concentrations was observed at low and high Cu^{2+} concentrations, despite the presence of Cu^{2+} enhanced CH_4-ECs (Figure 6).

<center>*<Figure 6>*</center>

3.4 Process microbiology

Phase contrast microscopy observations revealed two different morphologies of *Methylomicrobium*-like bacteria depending on the operational conditions tested. Agitation rates of 600 rpm favored rod-shaped bacteria under low Cu^{2+} concentrations regardless of salinity, while agitation rates of 300 rpm supported the predominance of sphered-shaped bacteria regardless of the Cu^{2+} and NaCl concentrations (Figure S1, supplementary materials). Pleomorphology (the ability of bacteria to alter their shape or size in response to environmental conditions) has been previously observed in *Methyloccocus* and *Methylosarcina* cultures, which are the closest phylogenetical lineages to the genus *Methylomicrobium* (Wise, 2001). *Methylomicrobium* cells at high Cu^{2+} concentrations appeared embedded in a dense matrix likely composed of the excreted ectoine (Figure7).

Methylomicrobium-like bacteria population was quantified by FISH analysis with the specific probe $M\gamma1004$ using the total DAPI-stained bacteria as a reference (Figure 7). The results showed that the genus *Methylomicrobium* accounted for more than 80% of the total bacterial population in most experimental runs. *Methylomicrobium* population in experimental runs 1, 3, 4 and 5 accounted for 89.2 % (*se* = 3.2), 102.9 % (*se* = 4.5), 104.9 % (*se* = 4.9) and 81.0 % (*se* = 3.4) of the total bacterial population, respectively. The biovolume fractions of *Methylomicrobium* in experimental runs 2 and 6 were slightly lower: 73.0 % (*se* = 3.5) and 69.1 % (*se* = 4.1), respectively. Biovolume shares higher than 100 % can be attributed to the fact that probe-hybridized cells can slightly increase their size/volume after hybridization. The high hybridization shares recorded suggested that the extreme conditions prevailing during the 6

<center>15</center>

experimental runs (alkaline pH and high salinity) prevented culture contamination with opportunistic microorganisms, which guarantees process robustness even under non-sterile conditions.

<Figure 7>

4. Conclusions

This study confirmed for the first time the feasibility of coupling ectoine production with the continuous abatement of dilute emissions of methane. NaCl concentration was the main factor influencing the accumulation of intra-cellular ectoine, with high salt concentrations inducing higher intra-cellular ectoine yields without compromising methane abatement. Apart from an unexpected increase in CH_4-ECs, the increase in Cu^{2+} concentration mediated the excretion of 20 % of the ectoine synthesized, thus enhancing its subsequent recovery. Process operation at high agitation rates damaged the bacterial population, with a subsequent decrease in both CH_4-ECs and ectoine yields. FISH analysis showed that *Methylomicrobium alcaliphilum 20Z* was the dominant microorganism regardless of the operational conditions as a result of the high pH and salinity prevailing in the culture broth. The promising results obtained in this study support the need for further research in order to implement CH_4 biorefineries for the production of ectoine (either in a process similar to the currently used by *Halomonas elongata*, namely "bio-milking", or by promoting the natural excretion of ectoine to the culture broth concentration at high Cu^{2+} concentrations) and open up a door to the development of a new generation of GHG biorefineries based on extremophile methanotrophs capable of creating value out of methane mitigation.

Acknowledgements

5. References

American Water Works Association, 2012. Standard Methods for the Examination of Water and Wastewater, American Water Works Association/American Public Works Association/Water Environment Federation.

But, S.Y., Khmelenina, V.N., Mustakhimov, I.I., Trotsenko, Y.A., 2013. Construction and Characterization of Methylomicrobium alcaliphilum 20Z Knockout Mutants Defective in Sucrose and Ectoine Biosynthesis Genes. Microbiology 82, 253–255. doi:10.1134/S0026261713020021

Cantera, S., Estrada, J.M., Lebrero, R., García-Encina, P.A., Muñoz, R., 2015. Comparative performance evaluation of conventional and two-phase hydrophobic stirred tank reactors for methane abatement: Mass transfer and biological considerations. Biotechnol. Bioeng. n/a–n/a. doi:10.1002/bit.25897

Cantera, S., Lebrero, R., García-encina, P.A., Mu, R., 2016a. Evaluation of the in fl uence of methane and copper concentration and methane mass transport on the community structure and biodegradation kinetics of methanotrophic cultures 171, 11–20. doi:10.1016/j.jenvman.2016.02.002

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

Chung, H.J., Bang, W. and Drake, M.A. (2006) Stress response of *Escherichia coli*. Compr Rev Food Sci Food Saf 5, 52–64.

Daims, H., Lücker, S., Wagner, M., 2006. daime , a novel image analysis program for microbial ecology and biofilm research. Environ. Microbiol. 8, 200–213. doi:10.1111/j.1462-2920.2005.00880.x

Duan, Z., Mao, S., 2006. A thermodynamic model for calculating methane solubility, density and gas phase composition of methane-bearing aqueous fluids from 273 to 523 K and from 1 to 2000 bar. Geochim. Cosmochim. Acta 70, 3369–3386. doi:10.1016/j.gca.2006.03.018

Eller, G., Stubner, S., Frenzel, P., 2001. Group-speci ¢ c 16S rRNA targeted probes for the detection of type I and type II methanotrophs by £ uorescence in situ hybridisation. FEMS Microbiol. Lett. 198, 91–97.

Estrada, J.M., Lebrero, R., Quijano, G., Pérez, R., Figueroa-González, I., García-Encina, P.A., Muñoz, R., 2014. Methane abatement in a gas-recycling biotrickling filter: Evaluating innovative operational strategies to overcome mass transfer limitations. Chem. Eng. J. 253, 385–393. doi:10.1016/j.cej.2014.05.053

European Environmental Agency, 2015. Atmospheric greenhouse gas concentrations (CSI 013/CLIM 052) - Assessment published Feb 2015. [WWW Document]. http://www.eea.europa.eu/data-and-maps/indicators/atmospheric-greenhouse-gas-concentrations-4/assessment.

ICH, E.W. group, 2005. Validation of analytical procedures: text and methodology q2(r1), in: International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use.

Intergovernmental Panel on Climate Change, 2014. Fifth assessment report: Climate change 2015, Synthesis Report. https://www.ipcc.ch/report/ar5/syr/ Accessed 12 December 2016

Kaluzhnaya, M., Khmelenina, V., Eshinimaev, B., Suzina, N., Nikitin, D., Solonin, a, Lin, J.L., McDonald, I., Murrell, C., Trotsenko, Y., 2001. Taxonomic characterization of new

alkaliphilic and alkalitolerant methanotrophs from soda lakes of the Southeastern

Transbaikal region and description of *Methylomicrobium buryatense* sp.nov. Syst. Appl.

Microbiol. 24, 166–176. doi:10.1078/0723-2020-00028

Kalyuzhnaya, M.G., Khmelenina, V., Eshinimaev, B., Sorokin, D., Fuse, H., Lidstrom, M.,

Trotsenko, Y., 2008. Classification of halo(alkali)philic and halo(alkali)tolerant

methanotrophs provisionally assigned to the genera *Methylomicrobium* and **Methylobacter**

and emended description of the genus *Methylomicrobium*. Int. J. Syst. Evol. Microbiol. 58,

591–596. doi:10.1099/ijs.0.65317-0

Khmelenina, N.V., Kalyuzhnaya, G.M., Starostina, G.N., Suzina, E.N., Trotsenko, A.Y., 2000.

Isolation and Characterization of Halotolerant Alkaliphilic Methanotrophic Bacteria from

Tuva Soda Lakes. Curr. Microbiol. 35, 257–261. doi:10.1007/s002849900249

Khmelenina, V.N., Kalyuzhnaya, M.G., Sakharovsky, V.G., Snzina, N.E., Trotsenko, Y. a.,

Gottschalk, G., 1999. Osmoadaptation in halophilic and alkaliphilic methanotrophs. Arch.

Microbiol. 172, 321–329. doi:10.1007/s002030050786

Khmelenina, V.N., Sakharovskiǐ, V.G., Reshetnikov, a S., Trotsenko, I. a, 2000. Synthesis of

osmoprotectors by halophilic and alkalophilic methanotrophs. Mikrobiologiia 69, 465–470.

Lang, Y ., Bai, L., Nan Ren, Y.,. Hua Zhang, Nagata L., 2011. Production of ectoine through a

combined process that uses both growing and resting cells of *Halomonas salina DSM

5928T*. Extremophiles, 15 (2011), pp. 303–310. doi.org/10.1007/s00792-011-0360-9.

López, J.C., Quijano, G., Souza, T.S.O., Estrada, J.M., Lebrero, R., Muñoz, R., 2013.

Biotechnologies for greenhouse gases (CH4, N2O, and CO2) abatement: state of the art and

challenges. Appl. Microbiol. Biotechnol. 97, 2277–2303. doi:10.1007/s00253-013-4734-z

Nikiema, J., Brzezinski, R., Heitz, M., 2007. Elimination of methane generated from landfills by

biofiltration: a review. Rev. Environ. Sci. Biotechnol. 6, 261–284. doi:10.1007/s11157-006-

9114-z

Pastor, J.M., Salvador, M., Argandoña, M., Bernal, V., Reina-Bueno, M., Csonka, L.N., Iborra, J.L., Vargas, C., Nieto, J.J., Cánovas, M., 2010. Ectoines in cell stress protection: Uses and biotechnological production. Biotechnol. Adv. 28, 782–801. doi:10.1016/j.biotechadv.2010.06.005

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. doi:10.1007/s00203-005-0042-z

Rocha-Rios J, Bordel S, Hernández S, Revah S. 2009. Methane degradation in two-phase partition bioreactors. *Chem. Eng. J.* **152**:289–292.

Semrau, J.D., Dispirito, A.A., Yoon, S., 2010. Methanotrophs and copper. FEMS Microbiol. Rev. 34, 496–531. doi:10.1111/j.1574-6976.2010.00212.x

Shchukin, V.N., Khmelenina V.N., Eshinimayev B. Ts., Suzina N. E., Trotsenko Y.A., 2011. Primary characterization of dominant cell surface proteins of halotolerant methanotroph *Methylomicrobium alcaliphilum* 20Z. Microbiology 80, 608. doi:10.1134/S0026261711050122

Stoecker, K., Dorninger, C., Daims, H., Wagner, M., 2010. Double labeling of oligonucleotide probes for fluorescence in situ hybridization (DOPE-FISH) improves signal intensity and increases rRNA accessibility. Appl. Environ. Microbiol. 76, 922–926. doi:10.1128/AEM.02456-09

Strong, P., Xie, S., Clarke, W.P., 2015. Methane as a resource: can the methanotrophs add value? Environ. Sci. Technol. 49, 4001–4018. doi:10.1021/es504242n

Strong, P.J., Kalyuzhnaya, M., Silverman, J., Clarke, W.P., 2016. Bioresource Technology A methanotroph-based biorefinery : Potential scenarios for generating multiple products from a

single fermentation. Bioresour. Technol. doi:10.1016/j.biortech.2016.04.099

Tanimura, K., Nakayama, H., Tanaka, T., Kondo, A., 2013. Ectoine production from lignocellulosic biomass-derived sugars by engineered *Halomonas elongata*. Bioresour. Technol. 142, 523–529. doi:10.1016/j.biortech.2013.05.004

Wise,M., McArthur V., Shimkets L., 2001. *Methylosarcina fibrata* gen. nov., sp. nov. and *Methylosarcina quisquiliarum* sp. nov., novel type I methanotrophs. *International Journal of Systematic and Evolutionary Microbiology* (2001), 51, 611–621

Table 1. Operational conditions evaluated during *Methylomicrobium alcaliphilum 20Z* cultivation in STRs for the optimization of CH_4 abatement and ectoine production

Experimental Run	Factors of study		
	NaCl (%)	Cu^{2+} (μM)	Agitation rate (rpm)
1	3	0.05	300
2	6	0.05	300
3	3	0.05	600
4	6	0.05	600
5	3	25	300
6	6	25	300

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Figure 1. Time course of the intra-cellular ectoine concentration at **a)** 3 % NaCl-300 rpm-0.05µM Cu^{2+} (continuous black line), 3 % NaCl-600 rpm-0.05µM Cu^{2+} (continuous grey line) and 3 % NaCl-300 rpm-25µM Cu^{2+} (dotted line) and **b)** 6 % NaCl-300 rpm-0.05µM Cu^{2+} (continuous black line), 6 % NaCl-600 rpm-0.05µM Cu^{2+} (continuous grey line) and 6 % NaCl-300 rpm-25µM Cu^{2+} (dotted line). ■, ● and ▲ represent the sampling times

Figure 2. Influence of the concentration of NaCl and Cu^{2+} and stirring rate on the steady state intra-cellular ectoine yield. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.

Figure 3. Time course of the extra-cellular ectoine concentration at **a)** 3 % NaCl-300 rpm-0.05µM Cu^{2+} (continuous black line) and 6 % NaCl-300 rpm-0.05µM Cu^{2+} (dotted line) **b)** 3 % NaCl-600 rpm-0.05µM Cu^{2+} (continuous black line) and 6 % NaCl-600 rpm-0.05µM Cu^{2+} (dotted line) **c)** 3 % NaCl-300 rpm-25µM Cu^{2+} (continuous black line) and 6 % NaCl-300 rpm-25µM Cu^{2+} (dotted line). The symbols ■ and ▲ represent the sampling times.

Figure 4. Influence of the concentration of NaCl and Cu^{2+} and stirring rate on the steady state concentrations of extra-cellular ectoine (black column), intra-cellular ectoine (grey column) and total ectoine (white column). Vertical lines represent standard deviations from replicates. Columns intra-groups with different letters were significantly different at $p<0.05$.

Figure 5. Influence of the concentration of NaCl and Cu^{2+} and stirring rate on the steady state CH_4 elimination capacity (CH_4-EC) (white column) and total CO_2 production (TPCO$_2$) (grey column). Vertical lines represent standard deviations from replicates. Columns intra-groups with different letters were significantly different at $p<0.05$.

Figure 6. Influence of the concentration of NaCl and Cu^{2+} and stirring rate on the steady state biomass concentration. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.

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Figure 7. Epifluorescence images (100×) of *Methylomicrobium* culture hybridized with Mγ1004 (Cy3-labeled) (2, 5, 8) in contrast to DAPI staining (1, 4, 7). The corresponding phase contrast image of each field of view is shown (3, 6, 9). Images 1-3 correspond to experimental run 1 (3% NaCl-300 rpm-low Cu^{2+}), images 4-6 correspond to experimental run 3 (3% NaCl-600 rpm-low Cu^{2+}), images 7-9 correspond to experimental run 6 (6% NaCl-300 rpm,-high Cu^{2+}). Images from experimental runs 2, 4 and 5 are not shown due to their similar characteristics to experimental runs 1, 3 and 6, respectively.

Figure 1
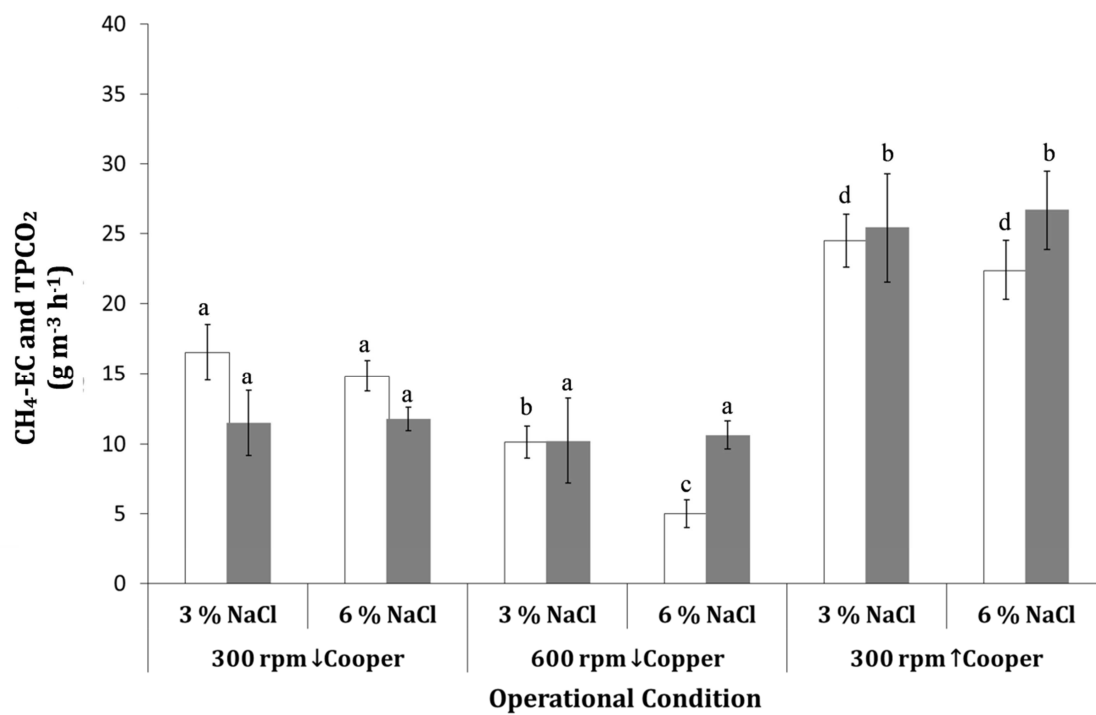
Figure 2

Figure 3

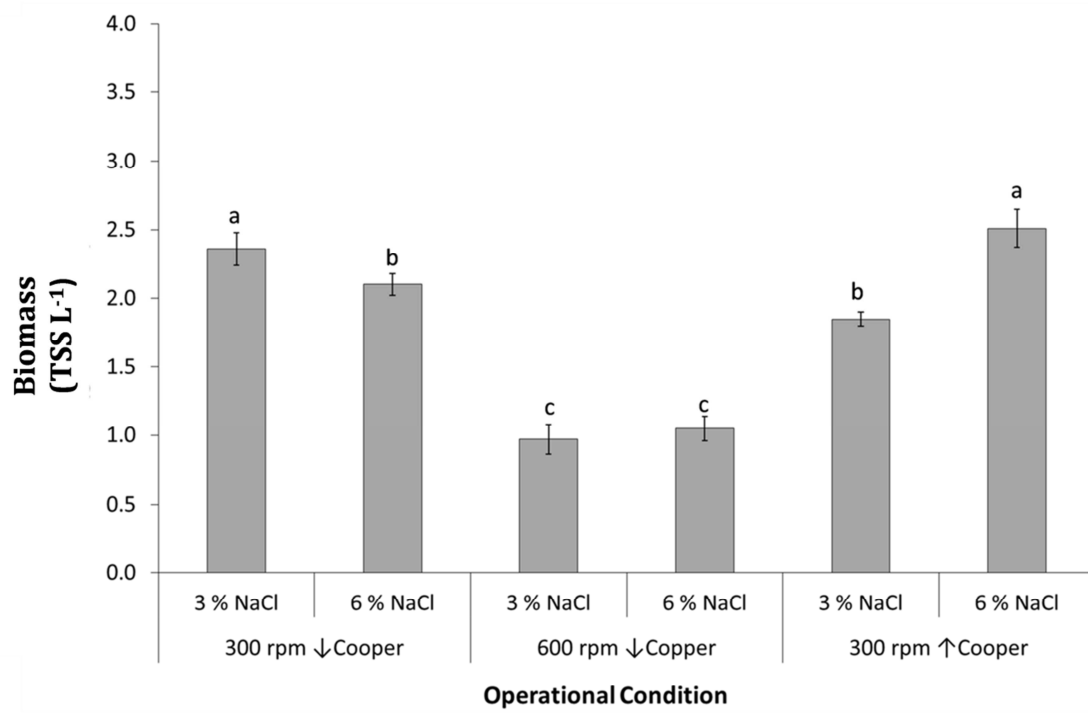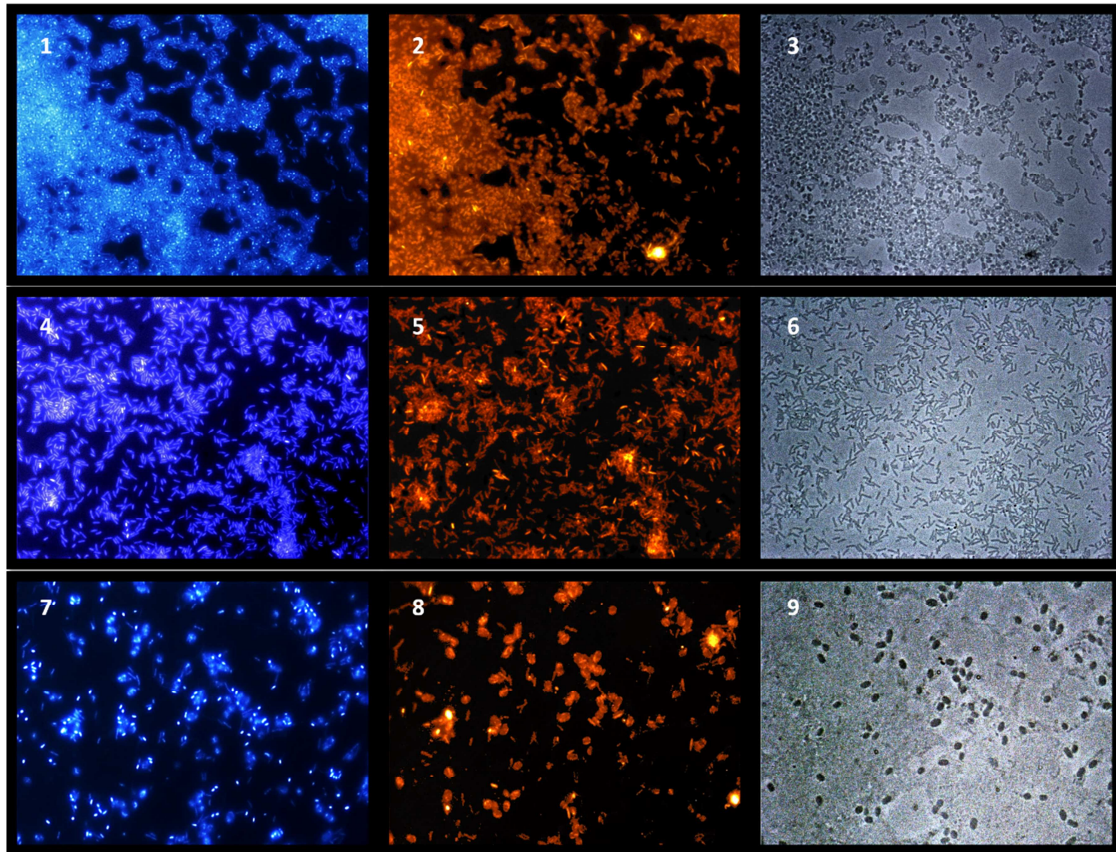**Figure 4**

Figure 5

Figure 6

Figure 7

Highlights:

a) Viability of coupling ectoine production with continuous methane mitigation.

b) High NaCl supported maximum ectoine production in the long-term operation.

c) High Cu^{2+} promoted the excretion of 20 % of the total ectoine synthesized.

d) Operational conditions avoided contamination and guaranteed process robustness.