Valorization of CH_4 emissions into high-added-value products: Assessing the production of ectoine coupled with CH_4 abatement

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Abstract:

This study assessed an innovative strategy for the valorization of dilute methane emissions based on the bio-conversion of CH_4 (the second most important greenhouse gas (GHG)) into ectoine by the methanotrophic ectoine-producing strain *Methylomicrobium alcaliphilum 20 Z*. The influence of CH_4 (2-20 %), Cu^{2+} (0.05-50µM) and NaCl (0-9 %) concentration as well as temperature (25-35 ºC) on ectoine synthesis and specific CH_4 biodegradation rate was evaluated for the first time. Concentrations of 20 % CH_4 (at 3 % NaCl, 0.05 µM Cu^{2+}, 25 ºC) and 6 % NaCl (at 4 % CH_4, 0.05 µM Cu^{2+}, 25 ºC) supported the maximum intra-cellular ectoine production yield (31.0 ± 1.7 and 66.9 ± 4.2 mg g biomass^{-1}, respectively). On the other hand, extra-cellular ectoine concentrations of up to 4.7 ± 0.1 mg L^{-1} were detected at high temperatures and Cu^{2+}concentrations (30 ºC and 50 µM), despite this methanotroph has not been previously classified as an ectoine-excreting strain. This research demonstrated the feasibility of the bio-conversion of dilute emissions of methane into high-added value products in an attempt to develop a sustainable GHG bioeconomy.

Keywords: Ectoine, Greenhouse Gas, Methane treatment, Methane biorefinery

1. Introduction

Methane (CH_4) is the second most important greenhouse gas (GHG) emitted nowadays as a result of its high global warming potential (25 times higher than that of CO_2) and emission rates (United States Environmental Protection Agency, 2015). Despite CH_4 can be used as an energy vector for electricity and heat generation when concentrations are higher than 20 %, more than 56 % of anthropogenic CH_4 emissions worldwide contain concentrations lower than 5 %. When applied to these dilute emissions (such as exhaust gases from landfills or coal mines), current CH_4 abatement technologies are neither environmentally friendly nor cost-effective (Avalos Ramirez et al., 2012).

Nowadays, the lack of a suitable approach to prevent the adverse environmental effects of CH_4 has encouraged both political initiatives to control these GHG emissions and an intensive research on novel strategies for CH_4 abatement (European Environmental Agency, 2015). In this regard, the biological abatement of dilute CH_4 emissions combined with the production of high-added value products represents, if properly tailored, a cost-effective alternative to mitigate CH_4 emissions. This CH_4 biorefinery approach would avoid the negative environmental effects of methane emissions while turning its treatment into a profitable process.

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is one of the most valuable microbial protective compounds against osmotic dehydration, as well as an efficient stabilizer for enzymes and nucleic acids (Pastor et al., 2010). This compound has attracted recent attention based on the high retail value that purified ectoine reaches in the cosmetic industry (approximately \$1300 kg^{-1}) (Strong et al., 2015). In 1999, Khmelenina et al. demonstrated that some moderate halophilic methanotrophs such as *Methylomicrobium*

alcaliphilum 20Z were able to produce and accumulate ectoine inside the cell (Kaluzhnaya et al., 2001; Khmelenina et al., 2000, 1999). These studies, conducted at high CH_4 concentrations, represented the first proof of the ability of CH_4-oxidizing bacteria to produce ectoine. However, little is known about the influence of environmental conditions on the bioproduction of this secondary metabolite when combined with the abatement of dilute CH_4 emissions. Furthermore, no studies on the production of extra-cellular ectoine (naturally excreted to the medium by specific excreting strains) by methanotrophs have been carried out to date.

The present study aimed at systematically elucidating the influence of CH_4, copper (Cu^{2+}) and NaCl concentrations, as well as temperature, on the extra and intra-cellular ectoine production using the strain *Methylomicrobium alcaliphilum 20Z*.

2. Materials and Methods

2.1. Chemicals and mineral salt medium

The mineral salt medium (MSM) used was a modified Brunner medium prepared according to Kalyuzhnaya et al. (2008) with a final pH of 9.1. NaCl and $CuCl_2 \cdot 2H_2O$ were supplemented to the MSM at the different concentrations tested (Table 1). All chemicals and reagents were purchased from Panreac (Barcelona, Spain) with a purity higher than 99.0 %. CH_4 was purchased from Abello-Linde, S.A. (Barcelona, Spain) with a purity of at least 99.5 %.

2.2. Microorganisms and inoculum preparation

64 The methanotrophic strain used in this study, *Methylomicrobium alcaliphilum 20Z*
65 (Kalyuzhnaya et al., 2008), was purchased from DSMZ (Leibniz-Institut).
66 *Methylomicrobium alcaliphilum 20Z* is an halophilic alkalitolerant methanotrophic strain
67 able to produce ectoine in the presence of NaCl (Khmelenina et al., 2000). Briefly, a 10×
68 dilution of the liquid *Methylomicrobium alcaliphilum 20Z* stock culture from DSMZ was
69 grown at 25 ºC in 120 mL glass bottles containing 90 mL of MSM at 3 % of NaCl and 0.05
70 µM Cu^{2+}. The bottles were closed with gas-tight butyl septa and metallic caps and 50 % v/v
71 of the air headspace was replaced by CH_4. The inoculum was ready to use in the batch
72 cultivation tests when a bacterial biomass concentration of 0.1 ± 0.06 g L^{-1} was achieved.

73 *2.3. Batch cultivation tests*

74 Five series of 13-day tests (TS) were performed in duplicate to evaluate the influence of
75 different environmental factors (CH_4, NaCl, Cu^{2+}, T) on the production of extra and intra-
76 cellular ectoine by *Methylomicrobium alcaliphilum 20 Z.* Sterile batch gas-tight reactors
77 (1.2 L) containing 190 mL of MSM and inoculated with 10 mL of the inoculum above
78 described (to an initial concentration of 0.05 ± 0.001 g L^{-1}) were used in each tests series.
79 The reactors were closed with gas-tight butyl septa and plastic screw caps. Unless
80 otherwise specified, all tests were initially supplied with a CH_4 headspace concentration of
81 25 g CH_4 m^{-3} (4 %), 3 % of NaCl, 0.05 µM Cu^{2+} and incubated at 25 ºC under a continuous
82 magnetic agitation of 600 rpm. The parameter evaluated in each specific test series was:
83 TS1: methane concentration (2, 4 and 20 %), TS2: copper concentration (0.05, 25 and 50
84 µM), TS3: NaCl concentration (0, 3, 6 and 9 %), and TS4: temperature (25, 30, 35 ºC). A
85 final test (TS5) combining the optimum conditions for ectoine production obtained in the

86 previous test series (20 % CH_4, 50 μM Cu^{2+}, 6 % NaCl and 30 ºC) was also carried out
87 (Table 1).

88 *<Table 1>*

Table 1. Cultivation conditions evaluated during *Methylomicrobium alcaliphilum 20Z* batch cultivation tests.

Test series (TS)	Operating conditions			
	CH_4 (%)	**Cu^{2+} (μM)**	**NaCl (%)**	**T (ºC)**
TS1	2, 4, 20	0.05	3	25
TS2	20	0.05, 25, 50	3	25
TS3	20	0.05	0, 3, 6, 9	25
TS4	20	0.05	3	25, 30, 35
TS5	20	50	6	30

89

90 Aliquots of pure CH_4 were supplied to the headspace of the reactors in TS1 using a 100 mL
91 gas tight syringe. Copper and NaCl were supplied by addition of the corresponding amount
92 of salt to the cultivation broths in TS2 and TS3, respectively, while the different
93 temperatures used in TS4 (25, 30 or 35º C) were maintained using thermostatic baths
94 (Digiterm-S-150 20).

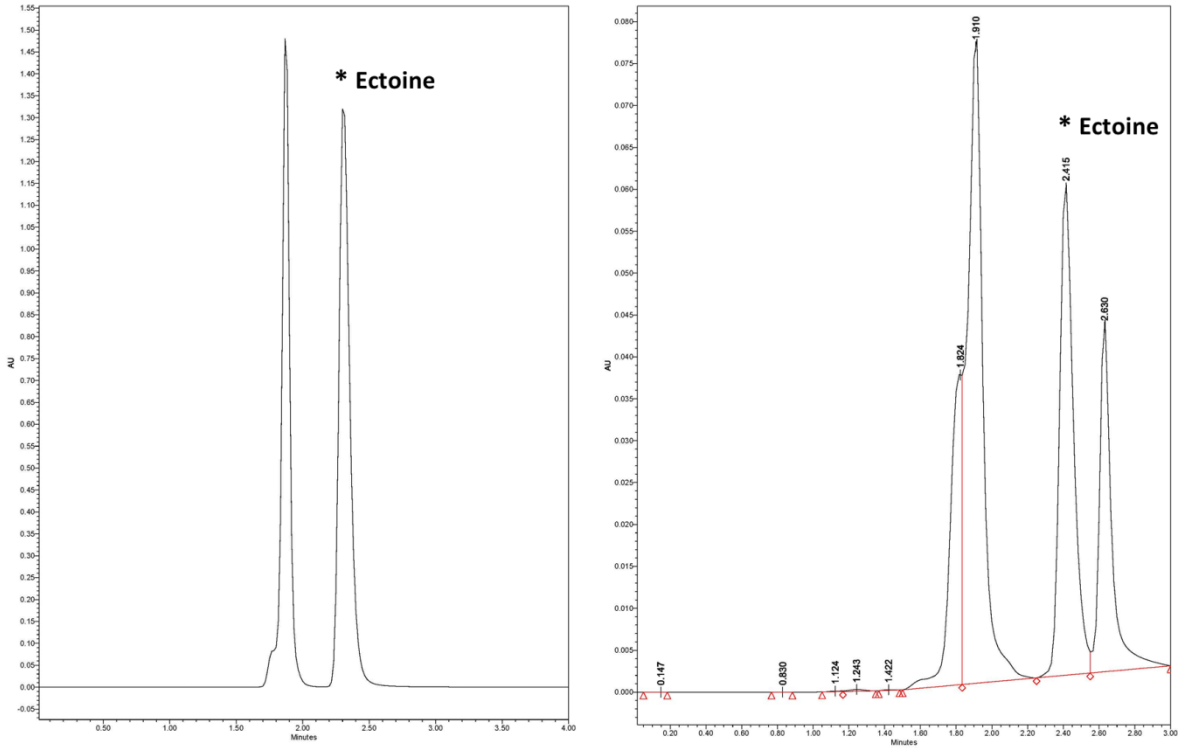
95 The O_2, CO_2 and CH_4 headspace concentrations were daily monitored. Aliquots of 10 mL
96 from the cultivation broth were also daily drawn with a liquid syringe to determine biomass
97 concentration and the intra and extra-cellular ectoine concentration. Biomass concentration
98 was estimated via culture absorbance measurements at 650 nm, which were previously

correlated to dry biomass concentrations (g L^{-1}) determined as total suspended solids (TSS) concentration.

2.4 Analytical procedures

The intra-cellular ectoine concentration was determined using 2 mL of cultivation broth centrifuged at 9000 g and 4 ºC for 15 min. Then, 2 mL of 80 % ethanol and 25 ± 5 mg of 0.1-mm-diameter zirconia/silica beads (BioSpec, Spain) were added to the Eppendorf tube containing the pellet. Microbial cells were then disrupted in a Mini-BeadBeater-16 (BioSpec, Spain) at 1048 g for 1 min and the suspension was kept overnight at room temperature (modified from Lang et al., 2011). The supernatant of these suspensions was used for ectoine analysis prior centrifugation at 9000 g and 4 ºC for 15 min and filtration through 0.22 μM filters (Filter-lab, Barcelona). The specific intra-cellular ectoine concentration (g ectoine g $biomass^{-1}$) was calculated using the TSS concentration (g L^{-1}) of the corresponding cultivation broth. An aliquot of 1 mL of cultivation broth was also drawn and filtered through 0.22 μM filters (Filter-lab, Barcelona) to measure the extra-cellular ectoine concentration. The concentration of ectoine was measured by HPLC-UV in a HPLC 717 plus auto-sampler (Waters, Bellefonte, USA) coupled with a UV Dual λ Absorbance detector (Waters, Bellefonte, USA detector) at 210 nm using a LC-18 AQ + C Supelcosil column (Waters, Bellefonte, EEUU) and a C18 AQ + pre-column (Waters, Bellefonte, EEUU). A phosphate buffer, consisting of 0.8 mM K_2HPO_4 and 6.0 mM Na_2HPO_4, was used as a mobile phase at 25 ºC 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) (Figure 1).

122 *<Figure 1>*



123

124 **Figure 1.** HPLC chromatograms a) Standard of ectoine at 100 mg L^{-1} in MSM b) ethanol
125 extracts of *Methylomicrobium alcaliphilum 20Z* cultivated at 3% NaCl, 25 ºC, 0.05 µM
126 Cu^{2+} and 4 % CH_4 in MSM.
127

128 CH_4, O_2 and CO_2 gas concentrations were determined in a Bruker 430 GC-TCD (Palo Alto,
129 USA) equipped with a CP-Molsieve 5A (15 m × 0.53 µm × 15 µm) and a CP-PoraBOND
130 Q (25 m × 0.53 µm × 10 µm) column. The oven, injector and detector temperatures were
131 maintained at 45 ºC, 150 ºC and 200 ºC, respectively. Helium was used as the carrier gas at
132 13.7 mL min^{-1}.

133 Culture absorbance measurements at 650 nm were conducted using a Shimadzu UV-2550
134 UV/Vis spectrophotometer (Shimadzu, Japan). TSS concentration was measured according
135 to standard methods (American Water Works Association, 2012).

136 *2.5. Data analysis*

137 The specific CH_4 degradation rate (SDR, g CH_4 $g^{-1}_{biomass}$ h^{-1}) was calculated from the slope
138 of the time course plot of methane concentration within the linear range in the batch
139 cultivation tests carried out. The statistical data analysis was performed using SPSS 20.0
140 (IBM, USA). The results are given as the average ± standard deviation. The homogeneity
141 of the variance of the parameters was evaluated using a Levene test. Significant differences
142 were analysed by ANOVA and post-hoc analysis for multiple group comparisons.
143 Differences were considered to be significant at $p \leq 0.05$.

144

145 **3. Results**

146 *3.1. Influence of cultivation conditions on intra-cellular ectoine production*

147 Intra-cellular ectoine reached its maximum concentration between days 5 and 7 of
148 cultivation regardless of the conditions tested. Subsequently, the concentration of intra-
149 cellular ectoine remained constant until the end of the assay (Figure 2a). No significant
150 difference was recorded in the intra-cellular ectoine concentration at 4 % CH_4, 3 % NaCl,
151 0.05 µM Cu^{2+} and 25 ºC in TS1-TS4, which confirmed the reproducibility and consistency
152 of the results here obtained. Both CH_4 and NaCl concentrations had a significant influence
153 on the production of intra-cellular ectoine (Figure 3). A CH_4 concentration of 20 %
154 supported maximum specific yields of 31.0 ± 1.7 mg ectoine g $biomass^{-1}$ by the end of the
155 cultivation, while the maximum yields obtained at CH_4 concentrations of 2 and 4 % were
156 9.9 ± 0.6 and 13.6 ± 3.8 mg ectoine g $biomass^{-1}$, respectively. A NaCl concentration of 6 %
157 was identified as the optimum value for the accumulation of intra-cellular ectoine, which

158 reached a maximum concentration of 66.9 ± 4.2 mg ectoine g biomass^{-1}. Higher or lower
159 salt concentrations supported lower ectoine yields (30.4 ± 7.5 mg ectoine g biomass^{-1} at 9
160 % NaCl; 12.5 ± 3.9 mg ectoine g biomass^{-1} at 3 % NaCl; 1.2 ± 0.5 mg ectoine g biomass^{-1} at
161 0 % NaCl). On the contrary, no significant effect ($p<0.05$) of temperature or Cu^{2+}
162 concentration was observed on the production of intra-cellular ectoine (Figure 3).

163 *<Figure 2>*

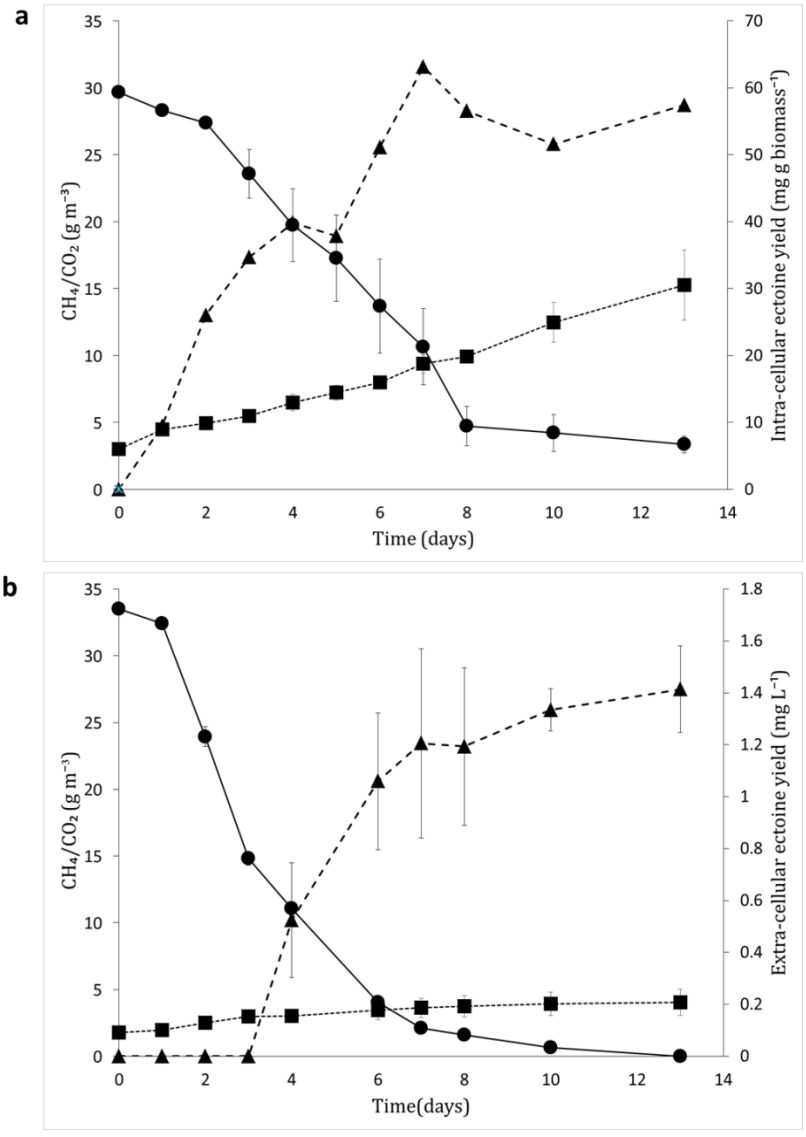


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Figure 2. Time course of the concentration of CH_4 (●, continuous line), CO_2 (■, dotted line) and intra-cellular (a) or extra-cellular (b) ectoine (▲, dashed line) at a) during *Methylomicrobium alcaliphilum 20Z* cultivation at 6 % NaCl, 0.05 µM Cu^{2+}, 25 ºC and 4 % CH_4, and b) at 3 % NaCl, 50 µM Cu^{2+}, 25 ºC and 4 % CH_4.

<Figure 3>

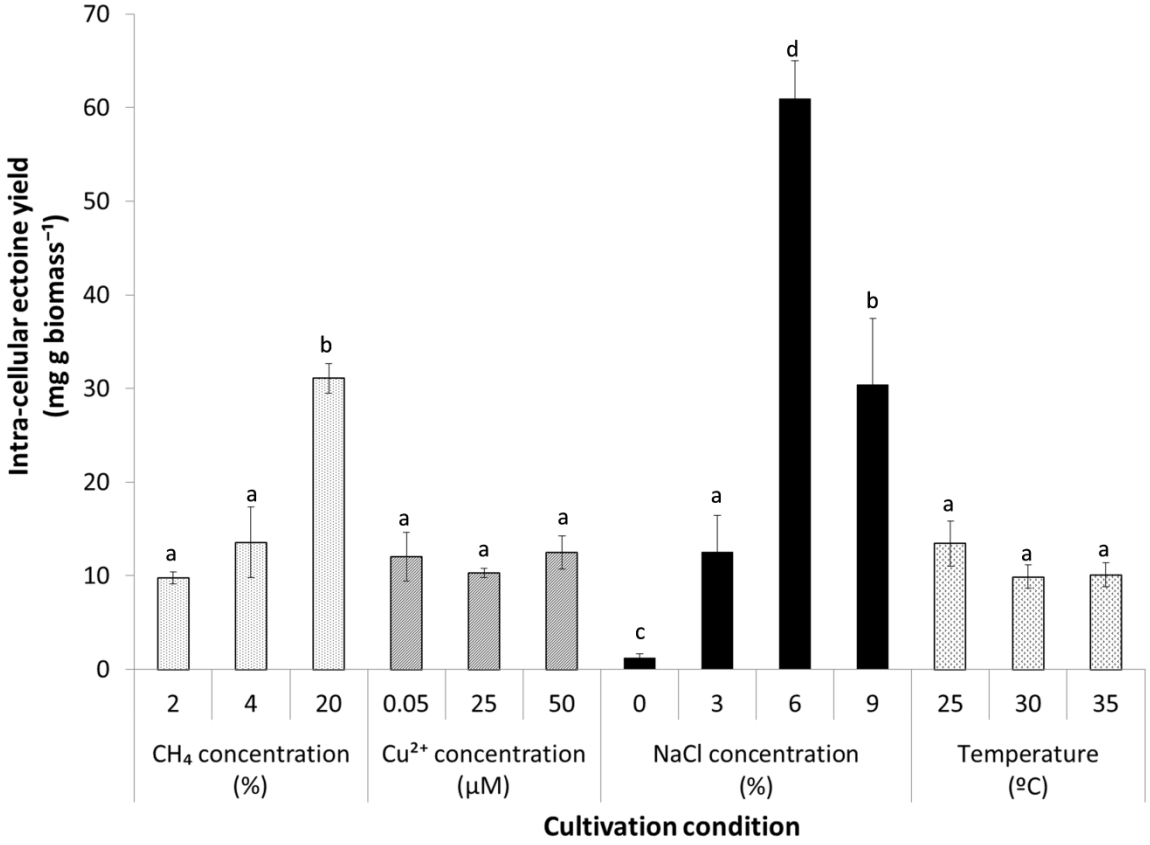


Figure 3. Maximum intra-cellular ectoine yield under different cultivation conditions. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.

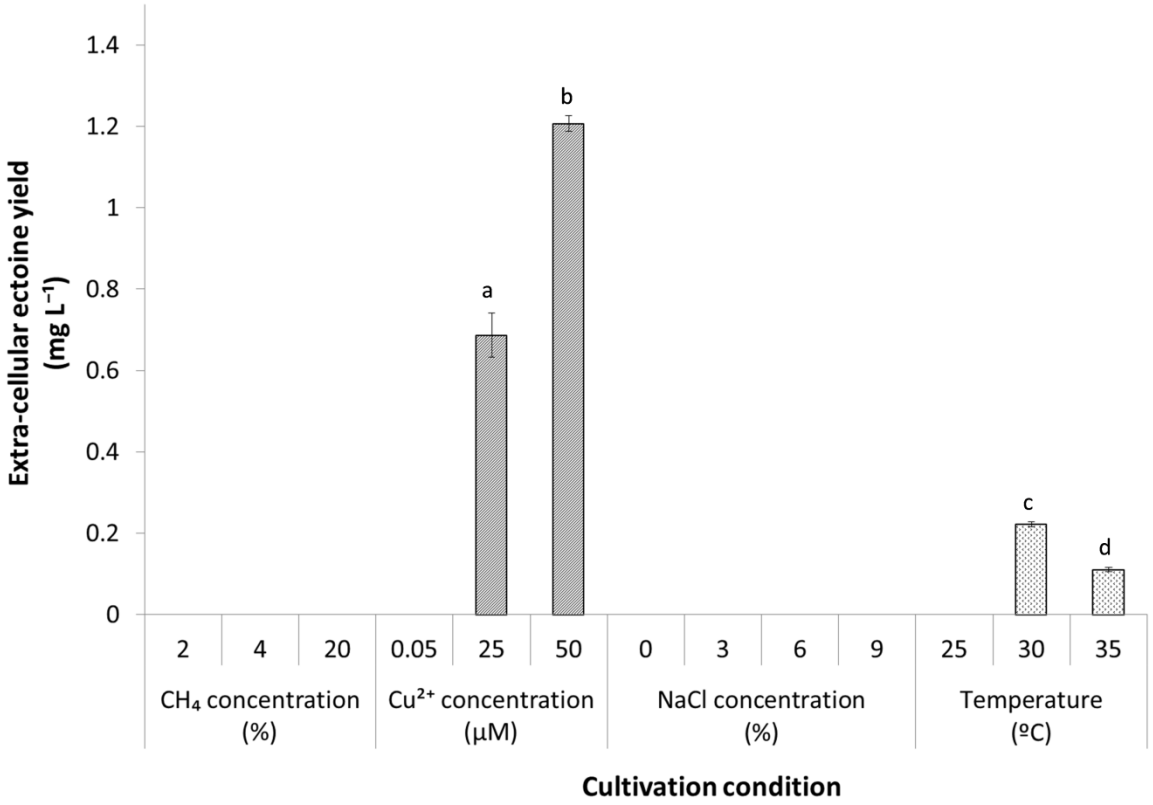
The maximum specific intra-cellular ectoine yields recorded at the different Cu^{2+} concentrations tested were 12.0 ± 2.6 mg ectoine g $biomass^{-1}$ at 0.05 µM Cu^{2+}, 10.3 ± 0.5 mg ectoine g $biomass^{-1}$ at 25 µM Cu^{2+} and 12.4 ± 0.7 mg ectoine g $biomass^{-1}$ at 50 µM

178 Cu^{2+}. Similarly, no significant effect of temperature on ectoine accumulation was observed
179 within the tested T range, with an average yield of 11.70 ± 1.1 mg ectoine g biomass^{-1}.

180 *3.2. Influence of cultivation conditions on ectoine excretion*

181 While no extra-cellular ectoine was detected during the 13 days of cultivation under the
182 different concentrations of CH_4 and NaCl tested, ectoine excretion was detected at high
183 temperature and Cu^{2+} concentration (Figure 4).

184 <*Figure 4*>



185

186 **Figure 4.** Extra-cellular ectoine excreted under different cultivation conditions. Vertical
187 lines represent standard deviations from replicates. Columns inter/intra-groups with
188 different letters were significantly different at $p<0.05$.

189 Ectoine excretion was observed by day 4 in tests supplemented with high Cu^{2+}
190 concentrations (Figure 2b). The maximum concentrations recorded were 0.7 ± 0.05 and 1.2
191 ± 0.01 mg extra-cellular ectoine L^{-1} at 25 and 50 µM of Cu^{2+}, respectively. Excretion of
192 ectoine was also observed at 30 and 35 ºC, although lower maximum concentrations were
193 detected under these particular cultivation conditions (0.2 ± 0.005 mg extra-cellular ectoine
194 L^{-1} at 30ºC and 0.1 ± 0.005 mg extra-cellular ectoine L^{-1} at 35ºC).

195 *3.3. Influence of cultivation conditions on the specific CH_4 degradation rate*

196 The results showed that a CH_4 headspace concentration of 20 % supported a significantly
197 ($p<0.05$) higher SDR (1.50 ± 0.08 g CH_4 h^{-1} g $biomass^{-1}$) compared to the SDRs recorded at
198 4 and 2 % of CH_4 (0.33 ± 0.05 and 0.29 ± 0.03 g CH_4 h^{-1} g $biomass^{-1}$, respectively) (Figure
199 5). On the contrary, the specific CH_4 oxidation rates decreased at higher NaCl
200 concentrations, with CH_4 SDRs of 0.05 ± 0.005, 0.22 ± 0.02, 0.34 ± 0.02 and 0.38 ± 0.02 g
201 CH_4 h^{-1} g $biomass^{-1}$ at 9, 6, 3 and 0 % NaCl, respectively. Neither temperature (25, 30 and
202 35 ºC supported SDRs of 0.34 ± 0.04, 0.30 ± 0.01 and 0.30 ± 0.01 g CH_4 h^{-1} g $biomass^{-1}$,
203 respectively) nor Cu^{2+} concentration (0.05, 25 and 50 µM of Cu^{2+} supported SDRs of 0.35
204 ± 0.01, 0.43 ± 0.01 and 0.37 ± 0.05 g CH_4 h^{-1} g $biomass^{-1}$, respectively) showed a
205 significant effect on the specific CH_4 degradation rate.

206 *<Figure 5>*

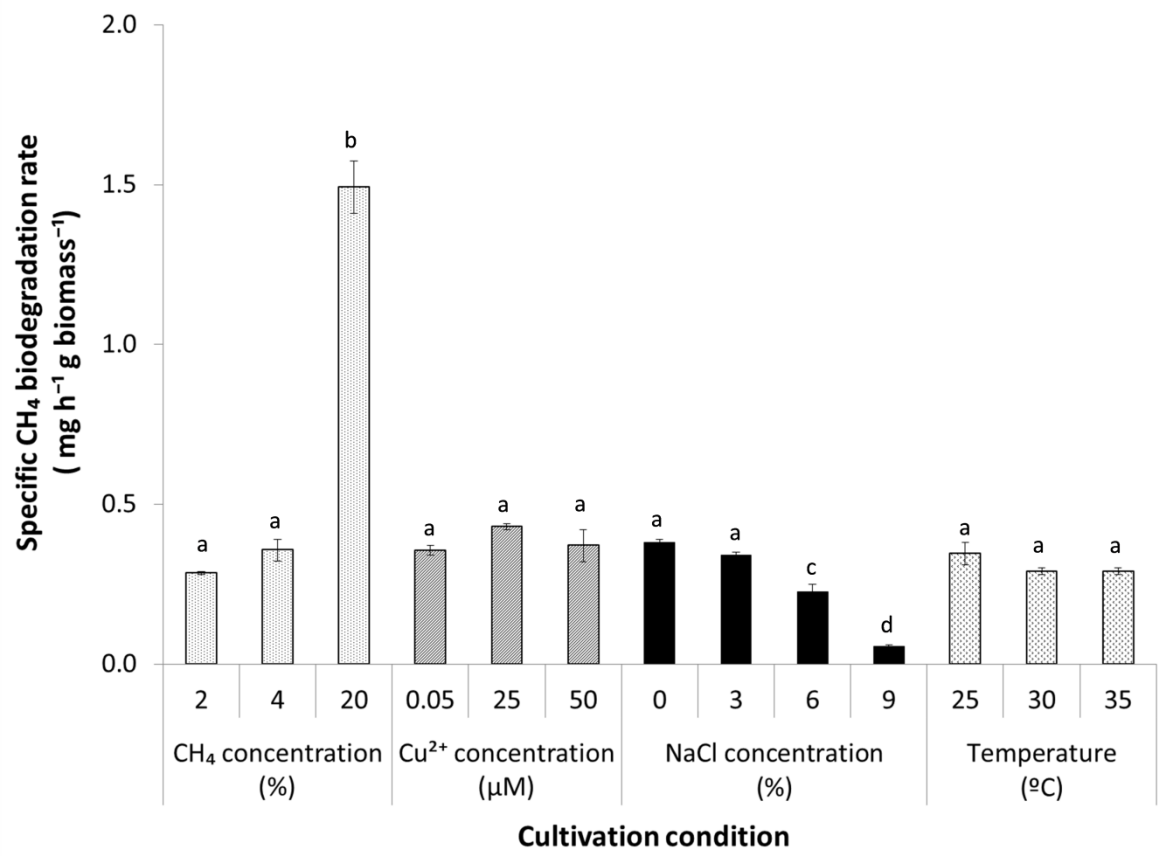


Figure 5. Specific CH_4 biodegradation rate under different cultivation conditions. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.

3.4. Production of extra and intra-cellular ectoine under optimum cultivation conditions

A final study was carried out combining the optimum parameters from previous tests TS1-TS4 in order to determine the production of extra and intra-cellular ectoine (20 % CH_4, 6 % NaCl, 30 ºC, 50 µM Cu^{2+}) (Table 2). These cultivation conditions promoted the excretion of ectoine to the extra-cellular medium (4.7 mg L^{-1}, which would correspond to 33.3 mg

217 ectoine g biomass^{-1}) and resulted in a high production of intra-cellular ectoine (40.7 ± 0.02

218 mg ectoine g biomass^{-1}).

219 *<Table 2>*

Table 2. Maximum values of ectoine concentration during *Methylomicrobium alcaliphilum 20Z* batch cultivation tests.

Test	Maximum intra-cellular ectoine	Maximum extra-cellular ectoine
	[Ectoine] (mg g biomass^{-1})	**[Ectoine]** (mg L^{-1})
TS1		
20% CH_4, 25ºC, 0.05µM Cu^{2+}, 3% NaCl	31.0 ± 1.7	N/D
TS2		
4% CH_4, 25ºC, 0.05µM Cu^{2+}, 6% NaCl	66.9 ± 4.2	N/D
TS3		
4% CH_4, 25ºC, 50µM Cu^{2+}, 3% NaCl	12.4 ± 0.7	1.2 ± 0.01
TS4		
4% CH_4, 30ºC, 0.05µM Cu^{2+}, 3% NaCl	10.6 ± 0.15	0.2 ± 0.005
TS5		
20 % CH4, 30ºC, 50µM Cu^{2+}, 6% NaCl	40.7 ± 0.02	4.7 ± 0.05

220

221

222 **4. Discussion**

223 This research investigated for the first time the effect of 4 environmental parameters (i.e.

224 CH_4 concentration, Cu^{2+} concentration, temperature and NaCl concentration) on the

production and excretion of ectoine and on the specific CH_4 degradation by *Methylomicrobium alcaliphilum 20 Z* in order to elucidate the optimum operational conditions to maximize ectoine production during the abatement of dilute CH_4 emissions.

A high concentration of CH_4 in the GHG emission significantly enhanced the production of intra-cellular ectoine likely due to an increase in substrate availability for the bacterial community, which induced high growth rates and therefore a high metabolite production (Estrada et al., 2014). In this sense, an increase in CH_4 concentration from 4 to 20 % enhanced the production of intra-cellular ectoine by a factor of 2.7 (from 9.9 ± 0.6 to 30.4 ± 7.5 mg ectoine g biomass^{-1}). However, no significant effect was observed on the accumulation of ectoine within the lower range of CH_4 concentrations tested (2 and 4 %). The key role of CH_4 concentration in the intra-cellular ectoine accumulation is in agreement with the results observed by Khemelenina et al. (2000), who recorded a maximum ectoine concentration of 200 mg ectoine g biomass^{-1} in *Methylomicrobium alcaliphilum 20 Z* under a CH_4 concentration of 50 % (v/v) in a MSM with 6 % of NaCl, 0.05 µM Cu^{2+} at 29 ºC (Khmelenina et al., 2000; Trotsenko et al., 2005). However, only dilute CH_4 emissions (<20 %) not suitable for energy recovery can be considered as a substrate of this novel CH_4 bio-refinery.

The salinity of the cultivation medium exhibited a positive effect on the intra-cellular ectoine yield up to a concentration of 6 % NaCl (66.9 ± 4.2 mg ectoine g biomass^{-1}), whereas higher salt concentrations resulted in lower ectoine yields (30.4 ± 7.5 mg ectoine g biomass^{-1} at 9 % of NaCl). Some authors have also observed a decrease in ectoine accumulation at increasing external salinity due to the regulation of the ectoine biosynthesis at the enzyme activity level (Reshetnikov et al., 2005). It is noteworthy that the

concentration of ectoine herein obtained at 6 % NaCl does not differ much from the values commonly encountered during the industrial production of ectoine using the glucose fermentative microorganism *Halomonas elongate* (yielding ectoine at an average value of 150.5 mg ectoine g biomass^{-1} when reused 9 times) (Strong et al., 2015). Nowadays, the process implemented at industrial scale (bacterial milking) involves the cyclic increase and decrease of the salt concentration in the cultivation broth up to 12 % NaCl. This process involving salt shocks increases reactor corrosion and hinders the downstream processing of ectoine due to the discontinuous nature of the production procedure and the high concentrations of salt. Alternatively, *Methylomicrobium alcaliphilum 20Z* can continuously synthesize a comparable high yield of ectoine in a less harsh medium coupled with CH_4 abatement from dilute emissions. Surprisingly, ectoine production was also observed in the absence of NaCl, although the concentrations detected were 55 times lower than the maximum ectoine yield recorded at 6 % of NaCl. The presence of a basal activity of the specific enzymes responsible for ectoine biosynthesis was likely related to the constitutive transcription of the ectoine gene cluster by this strain as previously observed by Khmelenina et al. (2000) and Reshetnikov et al. (2006). Likewise, Reshetnikov et al. (2006) confirmed that the optimum temperature for the enzymes catalyzing the key reactions of ectoine biosynthesis in *Methylomicrobium alcaliphilum 20Z* was 20 ºC, while temperatures higher than 30 ºC inhibited this reaction. However, no pernicious effect of temperature on the production of intra-cellular ectoine was observed in the present study. Moreover, no significant differences occurred among the ectoine yields recorded in the cultivation media containing different Cu^{2+} concentrations.

Ectoine excreting bacterial strains can accumulate ectoine and excrete it into the cultivation medium, thus enhancing the economics of the industrial production process of ectoine (Lang et al., 2011). Up to date, *Methylomicrobium alcaliphilum 20Z* has never been described as a strain able to excrete ectoine to the extra-cellular medium (Trotsenko et al., 2005). Whereas no extra-cellular ectoine was detected at the tested concentrations of NaCl and CH_4, cultivation at high temperatures and high Cu^{2+} concentrations promoted the excretion of a significant fraction of the intra-cellular ectoine. This excretion could be associated to the activation of unspecific channels or specific transporters able to excrete ectoine as a result of passive diffusion or an active transport of the cation Cu^{2+}. A research carried out by Schubert et al. (2007) observed that a transgenic *E.coli* genetically modified with the genes for ectoine biosynthesis, *ectABC*, from the halophilic bacterium *Chromohalobacter salexigens*, was able to excrete ectoine via expression of a specific transporter (Schubert et al., 2007). In this context, the presence of high Cu^{2+} concentrations in the cultivation medium could affect the activation of some specific transporters. Gram negative bacteria can express the tubular trans-membrane proteins porins, which allow the diffusion of small solutes and constitute a likely route for the simultaneous uptake of unchelated Cu^{2+} and excretion of intra-cellular ectoine in methanotrophs (Balasubramanian et al., 2011). Another plausible explanation for the excretion of intra-cellular ectoine might be the increase in cell membrane permeability, which is affected by temperature as observed by Kropinski et al. (1987) .

Along with the optimization of ectoine production, the maintenance of an efficient CH_4 abatement from dilute emissions of this GHG is also of key importance in the context of climate change mitigation. Hence, the highest SDRs were obtained at a CH_4 concentration

of 20 % as a result of the enhanced gas-liquid concentration gradients, which likely induced higher aqueous CH_4 concentrations and therefore higher specific growth rates (Cantera et al., 2016). However, no SDR enhancement was observed in the low range of CH_4 concentrations (2 and 4 %), as previously reported by Cantera et al. (2016). High salt concentrations negatively affected methane biodegradation in *Methylomicrobium alcaliphilum 20Z*, the SDR obtained at 9 % NaCl being 7.7 times lower than that obtained at 3 % NaCl. This strain is an halotolerant alkaliphilic methanotroph (Khmelenina et al., 1997) that can tolerate higher salt concentrations than other methanotrophs, but highly saline environments are not its optimum habitat. Thus, despite higher NaCl concentrations mediated the highest ectoine yields (6 and 9 %), a reduced CH_4 abatement performance was recorded. On the other hand, temperature and Cu^{2+} did not affect the SDR.

Finally, the optimum conditions (30ºC, 50 µM Cu^{2+}, 20 % of CH_4 and 6 % of NaCl) were combined in a test in order to maximize both extra and intra-cellular ectoine production. In this particular assay, the production of extra-cellular ectoine was 4 times higher than that obtained in TS2 (25ºC, 50 µM Cu^{2+}, 4 % of CH_4 and 3 % of NaCl), while the intra-cellular ectoine yield was lower than that recorded in TS3 (6 % of NaCl, 0.05 µM Cu^{2+} and 4 % of CH_4). Thus, the combination of these parameters favored the excretion of 44.4 % of the total intra-cellular ectoine produced. The total (intra-cellular + extra-cellular) ectoine produced would account for 73 mg ectoine g biomass^{-1} if not excreted, which was similar to the intracellular ectoine concentration (66.9 ± 4.2 mg gbiomass^{-1}) in TS3 at 6 % NaCl.

In summary, a proper selection of the environmental parameters (temperature, Cu^{2+}, NaCl and CH_4 concentration) for *Methylomicrobium alcaliphilum 20Z* cultivation is crucial to simultaneously maximize both the intra-cellular production and excretion of ectoine and

316 CH_4 abatement. The promising results here obtained support the further development of
317 CH_4 biorefineries capable of creating value out of GHG mitigation using extremophilic
318 methanotrophs.

319

320 ## 5. Acknowledgements

321 This research was supported by the Spanish Ministry of Economy and Competitiveness
322 (CTM2015-70442-R project and Red NOVEDAR), the European Union through the
323 FEDER Funding Program and the Regional Government of Castilla y León (PhD Grant
324 contract Nº E-47-2014-0140696 and UIC71).

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Table 1.

Table 1. Cultivation conditions evaluated during *Methylomicrobium alcaliphilum 20Z* batch cultivation tests.

Test series (TS)	Operating conditions			
	CH_4 (%)	Cu^{2+} (µM)	NaCl (%)	T (ºC)
TS1	2, 4, 20	0.05	3	25
TS2	20	0.05, 25, 50	3	25
TS3	20	0.05	0, 3, 6, 9	25
TS4	20	0.05	3	25, 30, 35
TS5	20	50	6	30

Table 2.

Table 2. Maximum values of ectoine concentration during *Methylomicrobium alcaliphilum 20Z* batch cultivation tests.

Test	Maximum intra-cellular ectoine	Maximum extra-cellular ectoine
	[Ectoine] (mg g biomass^{-1})	**[Ectoine]** (mg L^{-1})
TS1		
20% CH_4, 25ºC, 0.05µM Cu^{2+}, 3% NaCl	31.0 ± 1.7	N/D
TS2		
4% CH_4, 25ºC, 0.05µM Cu^{2+}, 6% NaCl	66.9 ± 4.2	N/D
TS3		
4% CH_4, 25ºC, 50µM Cu^{2+}, 3% NaCl	12.4 ± 0.7	1.2 ± 0.01
TS4		
4% CH_4, 30ºC, 0.05µM Cu^{2+}, 3% NaCl	10.6 ± 0.15	0.2 ± 0.005
TS5		
20 % CH4, 30ºC, 50µM Cu^{2+}, 6% NaCl	40.7 ± 0.02	4.7 ± 0.05

Figure 1.

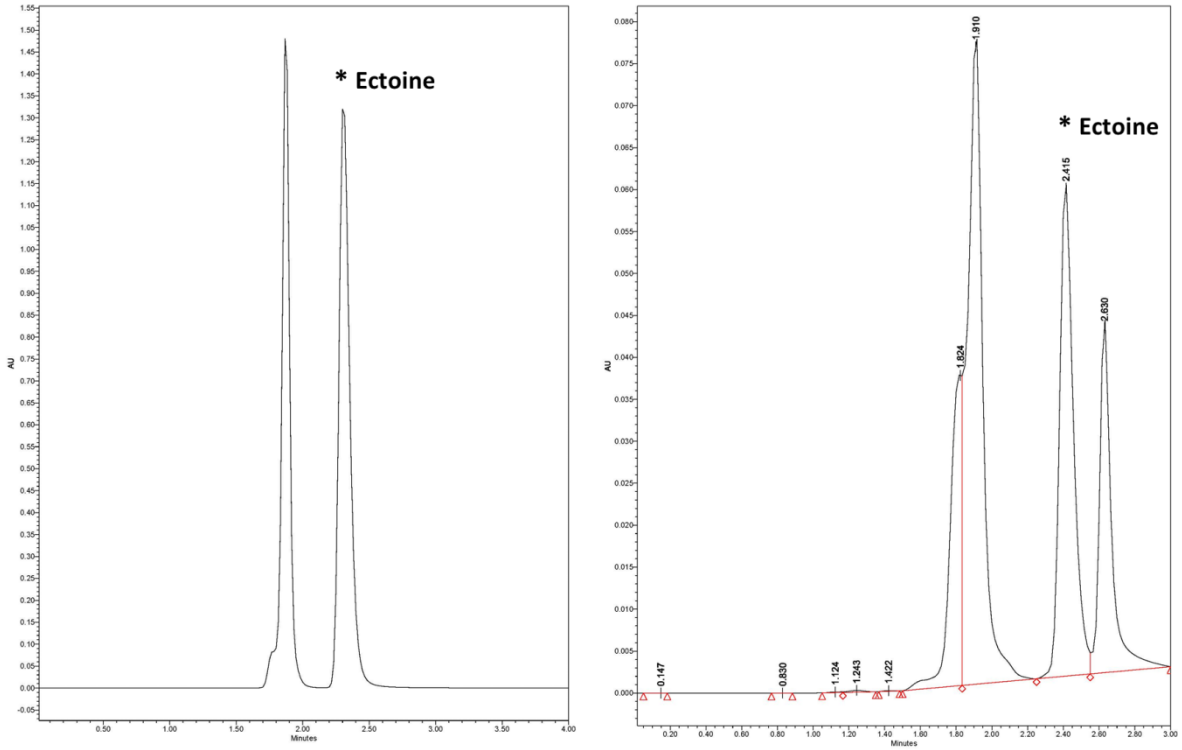

Figure 1. HPLC chromatograms a) Standard of ectoine at 100 mg L^{-1} in MSM b) ethanol extracts of *Methylomicrobium alcaliphilum 20Z* cultivated at 3% NaCl, 25 ºC, 0.05 µM Cu^{2+} and 4 % CH_4 in MSM.

Figure 2.

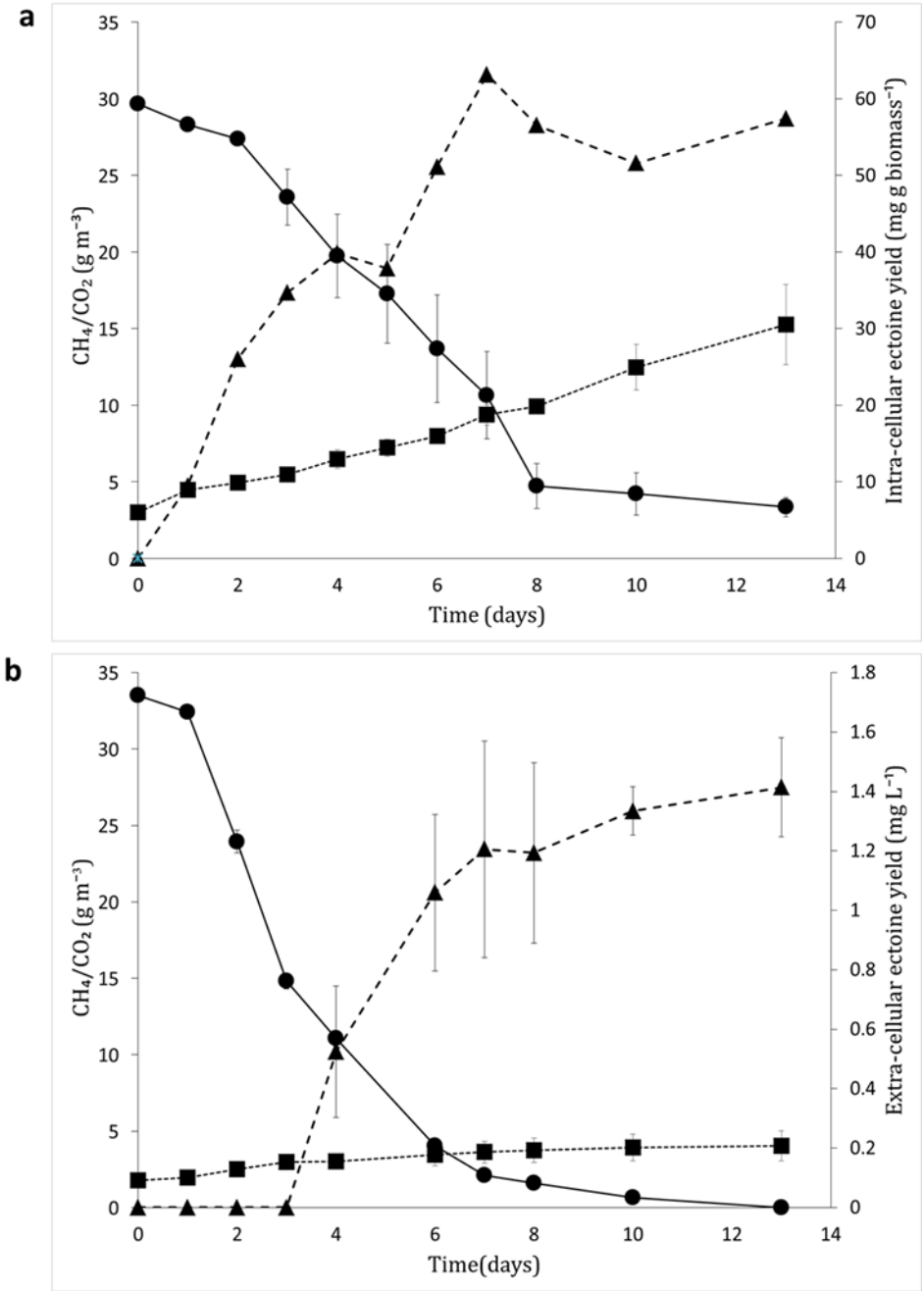

Figure 2. Time course of the concentration of CH_4 (●, continuous line), CO_2 (■, dotted line) and intra-cellular (a) or extra-cellular (b) ectoine (▲, dashed line) at a) during *Methylomicrobium alcaliphilum 20Z* cultivation at 6 % NaCl, 0.05 µM Cu^{2+}, 25 ºC and 4 % CH_4, and b) at 3 % NaCl, 50 µM Cu^{2+}, 25 ºC and 4 % CH_4.

Figure 3.

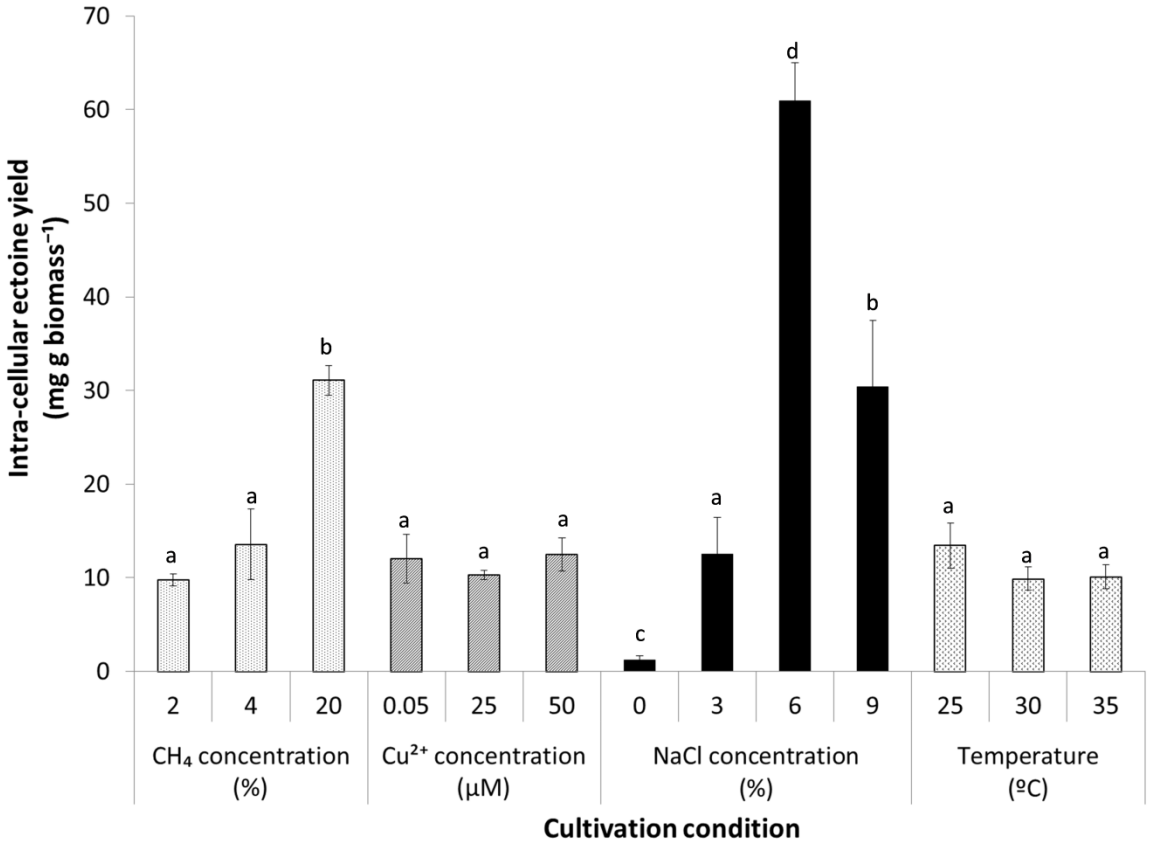

Figure 3. Maximum intra-cellular ectoine yield under different cultivation conditions. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.

Figure 4.

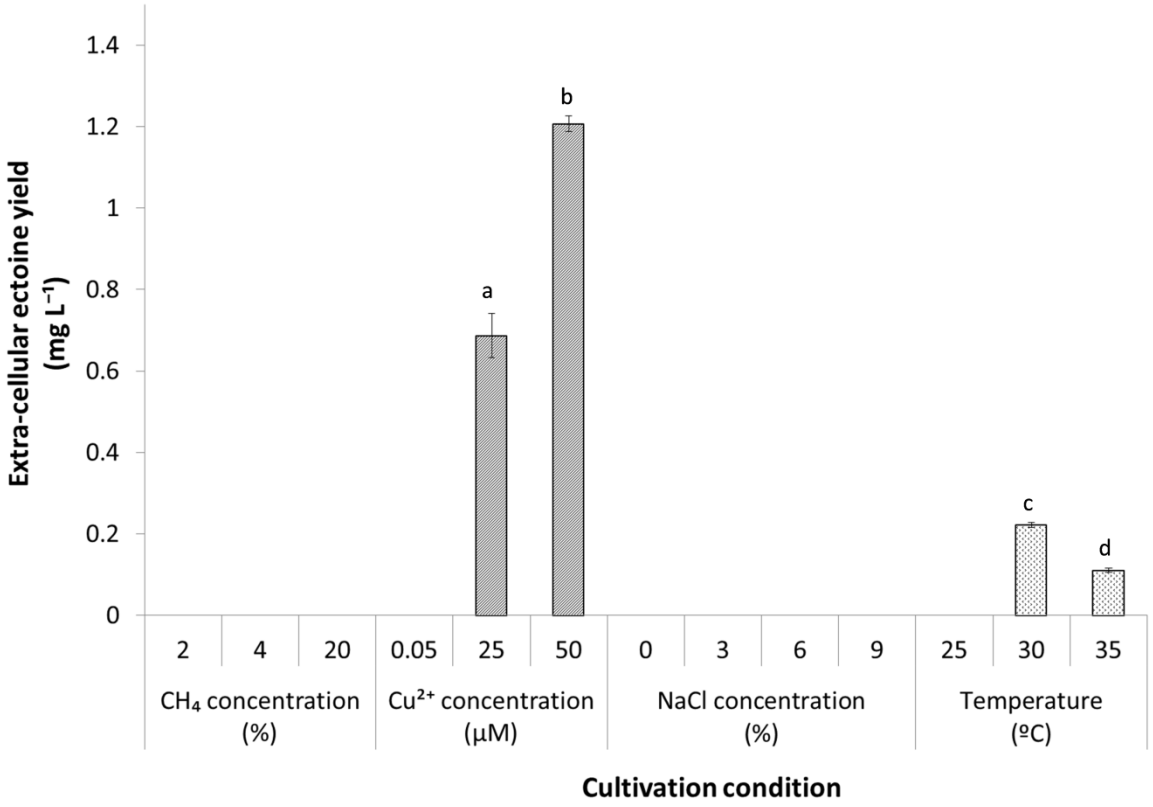

Figure 4. Extra-cellular ectoine excreted under different cultivation conditions. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.

Figure 5.

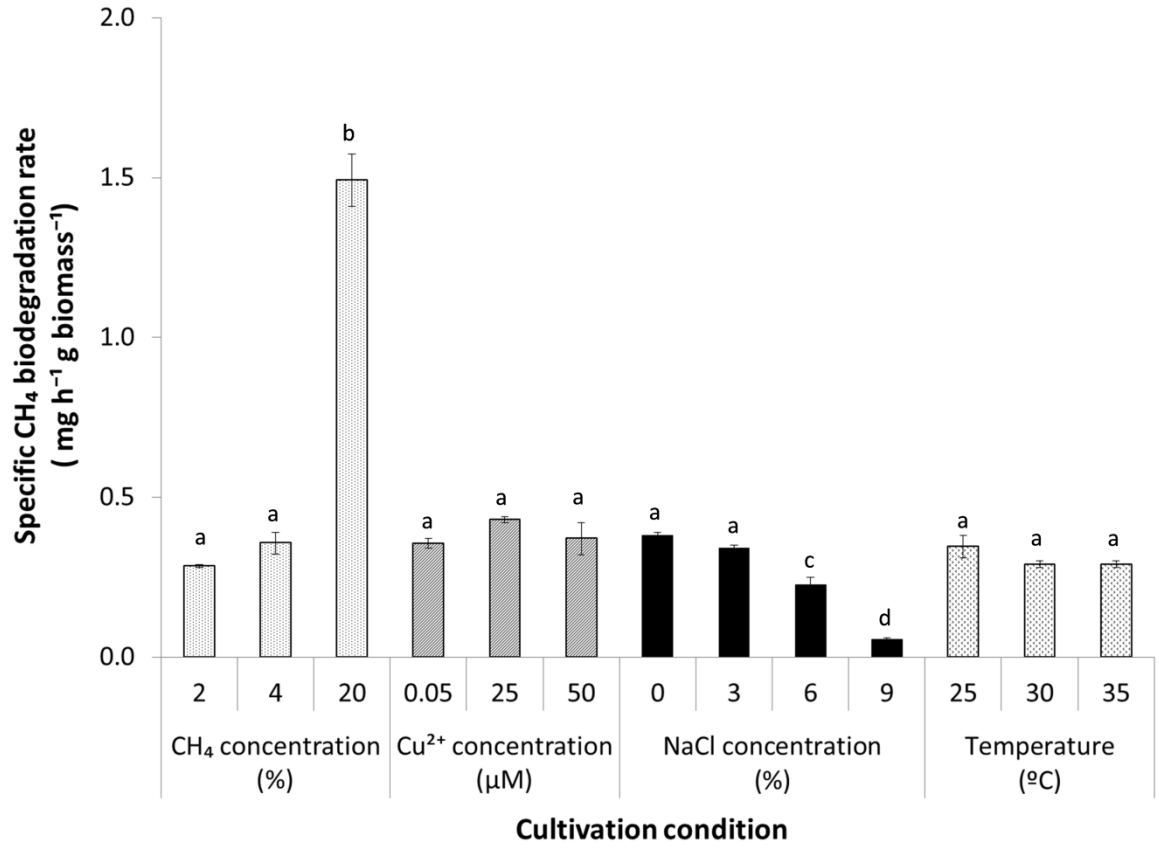

Figure 5. Specific CH_4 biodegradation rate under different cultivation conditions. Vertical lines represent standard deviations from replicates. Columns inter/intra-groups with different letters were significantly different at $p<0.05$.