Ectoine *bio-milking* in methanotrophs: A step further towards methane-based bio-refineries into high added-value products.

Short communication

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Abstract:

This communication showed for the first time that the methanotrophic strain *Methylomicrobium alcaliphilum 20Z* can efficiently synthesize and excrete (through a tailored *bio-milking process*) ectoine under continuous mode using methane as the sole energy and carbon source. First, three consecutive 50 h fed batch fermentations consisting of alternating high salinity (6% NaCl for 24h) and low salinity (0 % NaCl for 24h) cultivation stages were carried out in triplicate to determine the influence of sudden modifications in media salinity on ectoine synthesis and excretion. The results demonstrated that *M. alcaliphilum 20Z* exhibited a rapid response to osmotic shocks, which resulted in the release of the accumulated ectoine under hyposmotic shocks and the immediate uptake of the previously excreted ectoine during hyperosmotic shocks. A second experiment was carried out under continuous cultivation mode in two sequential stirred tank reactors operated at NaCl concentrations of 0 and 6 %. Cells exhibited a constant intra-cellular ectoine concentration of 70.4 ± 14.3 mg g biomass^{-1} along the entire operation period when cultivated at a NaCl concentration of 6%. The centrifugation of the cultivation broth followed by a hyposmotic shock resulted in the excretion of ~ 70 % of the total intra-cellular ectoine. In brief, this research shows the feasibility of the continuous bioconversion of diluted CH_4 emissions into high added-value products such as ectoine, which can turn greenhouse gas abatement into a sustainable and profitable process.

Keywords: Ectoine, *bio-milking*, methane abatement, methane biorefinery, *Methylomicrobium*

1. Introduction

The steady rise in the average temperature of the Earth within the last decades and its associated detrimental impacts on the environment have resulted in an increasing concern on global warming, which is triggering the enforcement of political initiatives for greenhouse gas (GHG) abatement [1]. Today, methane (CH_4) is the second most important GHG emitted worldwide based on its high global warming potential (25 times higher than that of CO_2), representing 12 % of the total GHG emission inventory [1,2]. The methane emitted by anthropogenic activities can be used for energy recovery when its concentration is higher than 30 % (v/v). However, more than 56 % of anthropogenic CH_4 emissions contain concentrations below 3 %, which are neither technical nor economically suitable for energy recovery [3]. These diluted emissions represent a threat to the environment [2,4,5] and their cost-efficient and environmentally friendly abatement is still unresolved [6,7]. In this context, the bioconversion of CH_4 into high added-value products such as ectoine has emerged as the most promising alternative for the control of diluted CH_4 emissions, which would turn GHG abatement into a sustainable and profitable process.

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is a compatible solute that maintains osmotic balance in a wide number of halotolerant bacteria [8]. Due to its high effectiveness as stabilizer of enzymes, DNA-protein complexes and nucleic acids, ectoine is likely one of the most valuable bioproducts synthesized by microorganisms. It retails in the pharmaceutical industry at approximately US$1000 kg^{-1} and its production at a global scale accounts for approx. 15000 tones $year^{-1}$ [9]. In this regard, biotechnologies for the production of ectoine have received increasing attention in the last two decades due to their simpler and highly specific product synthesis in comparison with chemical processes [8]. However, nowadays ectoine is only produced at industrial scale by the γ-*Proteobacteria Halomonas elongata*. The industrial ectoine production process -*bio-milking*- consists of a long fed-batch fermentation at varying NaCl concentrations. Thus, *H. elongata* is initially cultivated at low salinity to produce high density cultures, transferred to a high salinity medium to promote ectoine biosynthesis [10] and finally exposed to a hyposmotic shock to induce the excretion of ectoine from the cell to the cultivation broth, where the product is collected for its downstream purification [8]. Nevertheless, this

process is costly due to the need for high quality carbon sources (often glucose, yeast and sodium glutamate), which reduces its cost effectiveness [11,12].

Recent studies performed in both batch and continuous bioreactors have demonstrated the ability of the methanotrophic bacterium *Methylomicrobium alcaliphilum 20Z* to synthesize ectoine using methane as the sole carbon and energy source [13–15]. The promising results obtained hitherto with methanotrophs encourage further research devoted to develop biotechnologies capable of creating value from this greenhouse gas. This study constitutes, to the best of our knowledge, the first proof of concept of ectoine production by *M. alcaliphilum 20Z* under the current industrial fed-batch and osmotic downshock *bio-milking* process using diluted CH_4 emissions as a feedstock.

2. Experimental

A fed-batch fermentation, consisting of three consecutive 50 h two-stage cultivations at 25 ºC and 600 rpm, was initially carried out in triplicate in sterile 1.2 L gas-tight reactors containing 300 mL of a mineral salt medium (MSM) specific for alkalophilic methanotrophs [16] and inoculated at 0.13 ± 0.01 g TSS L^{-1} (Table S1). The two-stage *M. alcaliphilum 20Z* cultivation involved an initial incubation at high salinity (6 % NaCl) for 24 hours to promote bacterial growth and ectoine accumulation followed by a biomass harvesting step and a final bacterial incubation for 24 hours in the absence of NaCl to promote the excretion of ectoine (TSI). Cells were harvested by centrifugation at 9000g for 10 min, washed twice with a 0.85 % NaCl solution and re-suspended in a NaCl free MSM prior cultivation in NaCl free MSM, while 80 mL of sterile 22.5 % NaCl MSM were added to the NaCl free MSM prior cultivation at 6 % in TS2 and 3. The headspace CH_4 concentration at the beginning of each incubation stage (6 and 0 % NaCl) was 50 g CH_4 m^{-3} (8 %). The concentration of ectoine and biomass was monitored at time 0, 1, 3, 5, 7, 9, 12 and 24 hours by drawing 10 mL of cultivation broth. More details related to the fed batch fermentation can be found in Text S1 and Table S1.

<Fig. 1>

A second experiment targeting the continuous bioconversion of CH_4 into ectoine using a *bio-milking* approach was carried out in duplicate for 30 days in two 1-L stirred tank reactors agitated via a Rushton turbine (Fig. 1). The high cell density growth reactor (R1) was operated at pH 9 and 0 % NaCl, while the ectoine accumulation reactor (R2) was operated at pH 9 and 6 % NaCl. Process operation based on two reactors was necessary to achieve high ectoine concentrations in R2 since a single stage cultivation at 6 % NaCl involves the adaptation of *M. alcaliphilum* with the subsequent reduction in the intracellular osmoprotectant concentration. Preliminary tests showed that maximum ectoine accumulation occurred at biomass retention times of 5 days [15]. Both reactors were initially inoculated at a concentration of 0.34 g L^{-1} of *M. alcaliphilum 20Z* and operated at a CH_4 loading rate of 53 g m^{-3} h^{-1}. R1 and R2 were supplied with 120 and 100 mL d^{-1} of MSM at 0 and 12 % NaCl, respectively, while 100 mL of cultivation broth from R1 were daily transferred to R2. Thus, 180 mL of cultivation broth from R2 were daily drawn for ectoine extraction by centrifugation (9000 g for 10 min) followed by biomass resuspension in 20 mL of NaCl-free medium and incubation for one hour at 300 rpm and 25 ºC. The concentrations of intra and extra-cellular ectoine and biomass were determined in R1, R2 and the concentrated biomass extraction broth. Gas samples for CH_4 and CO_2 analysis were daily drawn at the inlet and outlet of the bioreactors using 100 µl gas-tight syringes (HAMILTON, Australia). A more detailed description of the reactor setup, the analytical procedures and the statistical analysis used for data treatment is provided in Text S2, S3 and S4, respectively.

3. Results and discussion

The fed-batch fermentation conducted confirmed that NaCl concentration exerted a positive effect on the intra-cellular accumulation of ectoine in *Methylomicrobium alcaliphilum 20Z* (Fig. 2) [13,14,17]. The specific intra-cellular ectoine concentrations reached 20.2 ± 4.2, 27.0 ± 2.0 and 25.8 ± 1.7 mg ectoine g biomass^{-1} at the end of the cultivation at high salinity in TS1, 2 and 3, respectively. The exposure of the cells to a low salinity medium resulted in a rapid excretion of the intra-cellular ectoine, which corresponded to 24.8 ± 2.2, 32.3 ± 2.7 and 29.1 ± 0.5 mg ectoine g biomass^{-1} in TS1, 2 and 3, respectively. Therefore, the hyposmotic shocks supported a complete ectoine excretion to the extracellular medium

(Fig. 2). A small fraction of the extra-cellular ectoine released to the cultivation broth (~27 %) was consumed by *M. alcaliphilum 20Z* during cultivation at 0 % NaCl (Fig. 2). When NaCl was supplied again to the cultivation broth containing the extracellular ectoine, the compatible solute was actively transported back into the bacterial cytoplasm. The concentrations of total ectoine (the sum of extra and intra-cellular ectoine) detected in low and high salinity media were not significantly different among the three two-stage cultivations.

<Fig. 2>

The evolution of the intra- and extra-cellular ectoine concentration during the first 64 h of experiment (Fig. 3) revealed that *M. alcaliphilum 20Z* was able to accumulate intra-cellular ectoine within the first 24 hours as a compatible solute to protect the cell against the osmotic stress caused by the high salinity of the cultivation broth. The decrease in NaCl concentration (at 25 h) induced a rapid excretion of the cytoplasmic ectoine to the cultivation broth. This immediate release of osmoprotectant solutes has been previously observed in *Halomonas* sp. when exposed to sudden hyposmotic shocks [8,10,18]. Moreover, a slight decrease in extra-cellular ectoine concentration was observed during the first hours of cultivation under low salinity, while no significant variation in the intra-cellular ectoine content was detected in this period. This finding was attributed to the use of ectoine as an readily biodegradable nitrogen and carbon source by *M. alcaliphilum 20Z* [13,14]. The restoration of NaCl salinity resulted in a gradual re-assimilation of the extra-cellular ectoine within the first 10 h of cultivation under high salinity. This study confirmed that *M. alcaliphilum 20Z* can uptake the extra-cellular ectoine previously released to avoid the additional expenses of anabolic energy required for a *de-novo* ectoine synthesis.

<Fig. 3>

3.2 Methane abatement in stirred tank reactors combined with ectoine *bio-milking*

Steady elimination capacities (ECs) and removal efficiencies (REs) of 11.1 ± 1.6 and 13.2 ± 1.2 g CH_4 m^{-3} h^{-1}, and 19.6 ± 2.9 and 22.5 ± 2.5 %, were recorded during the continuous CH_4 treatment in R1 and R2, respectively (table 1). Methane has an inherently low

solubility in water due to its hydrophobic nature (dimensionless CH_4 partition coefficient in water of 30 at 25 ºC[19]), which often entails a low CH_4 availability to the microbial community. Moreover, CH_4 solubility in water decreases at higher cultivation broth salinities, further limiting the mass transfer of CH_4 from the emission to the *M. alcaliphilum* culture [20]. However, no significant differences between the steady state CH_4-ECs and REs were recorded at low and high salt concentrations. Likewise, despite previous studies have observed an increase in the total CO_2 production ($TPCO_2$) at higher salinities [15], no statistical difference was encountered in this specific study (13.6 ± 2.9 and 14.10 ± 1.8 g CO_2 m^{-3} h^{-1} in R1 and R2, respectively). Thus, no significant effect of NaCl concentration neither on growth nor on CH_4 biodegradation activity was here recorded for *M. alcaliphilum 20Z*.

Table 1. Methane abatement performance in R1 and R2

Reactor	Elimination capacity (g m^{-3} h^{-1})	Total CO_2 Production (g m^{-3} h^{-1})	Removal Efficiency (%)
R1 (0% NaCl)	11.1 ±1.6[a]	13.6 ±2.9[b]	19.6 ± 2.9[c]
R2 (6% NaCl)	13.2 ±1.2[a]	14.1 ±1.8[b]	22.5 ±2.5[c]

Mean values within a column followed by different superscript letters were significantly different at $p<0.05$. Means±SD

During the 20 days of steady state operation neither intra- nor extra-cellular ectoine were detected in R1. The hyperosmotic shock in R2 induced a constant intra-cellular ectoine yield along the operation of 70.4 ± 14.3 mg g biomass^{-1} (corresponding to 42.44 ± 10.3 mg L^{-1}). Extra-cellular ectoine was also detected in the cultivation broth of R2 (4.3± 2.2 mg L^{-1}), probably due to a cell disruption mediated by the hyperosmotic shocks and the stirring shear stress. The hyposmotic shock after biomass harvesting and concentration resulted in intra-cellular ectoine concentrations of 30.3± 7.5 mg L^{-1} and extra-cellular concentrations of 253.4 ± 55.1 mg L^{-1} (Fig. 4). Therefore, the downstream operation was able to harvest 70.4 ± 24.6 % of ectoine accumulated in R2. This finding confirmed the hypothesis that *M. alcaliphilum 20Z* is able to release its compatible solutes in response to hyposmotic shocks

similarly to the industrial process used with *H. elongata*. The *bio-milking* strategy here investigated did not cause a significant damage in cell integrity, as shown by the comparable biomass concentrations before (0.61 ± 0.05 g L^{-1}) and after the hyposmotic shock (0.58 ± 0.07 g L^{-1}), which represented a survival rate of ~100 %.

<Fig. 4>

The production yields reported in literature are higher than those recorded for *M. alcaliphilum 20Z* in our study. For instance, the genus *Brevibacterium* accumulates 150-160 mg g biomass^{-1} depending on the specie, while ectoine yields of 155-200 mg g biomass^{-1} have been recorded in *Halomonas* sp.. Higher yields can be achieved by genetically modified microorganisms (*Escherichia coli* DH5α can synthesize up to 400-540 mg g biomass^{-1}) [8,11,12,21–23]. Nevertheless, *M. alcaliphilum 20Z* exhibits a superior environmental performance based on its ability to produce ectoine from dilute CH_4 emissions, with a concomitant mitigation of climate change.

4. Conclusions

The experiments performed demonstrated that the methanotrophic strain *M. alcaliphilum 20Z* can efficiently synthesize and excrete ectoine into the medium using methane as the sole energy and carbon source through the industrial process *bio-milking*. *M. alcaliphilum 20Z* was able to rapidly respond to hyposmotic shocks by rapidly releasing the accumulated solutes, and to hyperosmotic shocks by re-assimilating the excreted compatible solutes. Thus, *M. alcaliphilum 20Z* represents a promising strain for industrial ectoine production in continuous bioreactors, *bio-milking* supporting a recovery of ~ 70 % of the total intra-cellular ectoine accumulated, and resulting in extracellular concentrations of 253.4 ± 55.1 mg L^{-1}. To the best of our knowledge, this study constitutes the first proof of concept of ectoine *bio-milking* coupled to the abatement from diluted CH_4 emissions. The optimization of this innovative process will boost the development of a new generation of GHG biorefineries based on extremophile methanotrophs capable of creating value out of methane mitigation.

5. Acknowledgements

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Figure captions

Fig. 1: Schematic diagram of the experimental setup: high cell density growth reactor (R1), ectoine accumulation reactor (R2), mineral salt media storage tanks (MSM), Gas sampling port (GSP), Liquid sampling port (LSP), gas streams (continuous line), liquid streams (discontinuous line).

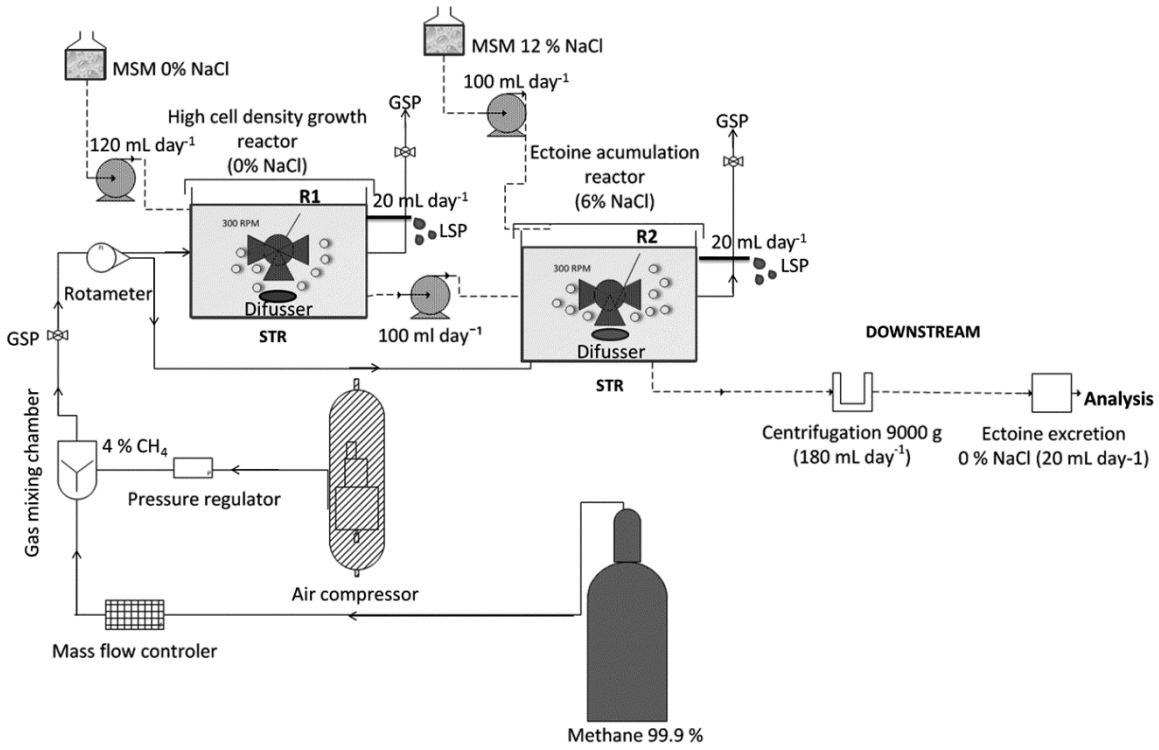
Fig. 2: Time course of the average intra (light grey column) and extra (dark grey column) -cellular ectoine concentration during the 3 sequential two-stage cultivations carried out in triplicate in batch gas tight reactors.

Fig. 3: Time course of the average intra (dashed line) and extra (continuous line) -cellular ectoine concentration during the initial 64 hours of experiment in batch gas tight reactors.

Fig. 4: Steady state intra-cellular (white column) and extra-cellular (grey column) ectoine culture broth concentrations in R2 and in the extracted broth after *bio-milking*. Vertical lines represent standard deviations from replicates.

Figure

Fig. 1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MSM 12 % NaCl
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GSP
High cell density growth reactor (0% NaCl)
120 mL day-1
Ectoine acumulation reactor (6% NaCl)
GSP
R1
20 mL day-1
LSP
300 RPM
R2
20 mL day-1
LSP
300 RPM
Rotameter
Difusser
STR
100 ml day-1
Difusser
STR
DOWNSTREAM
GSP
Analysis
Centrifugation 9000 g (180 mL day-1)
Ectoine excretion 0 % NaCl (20 mL day-1)
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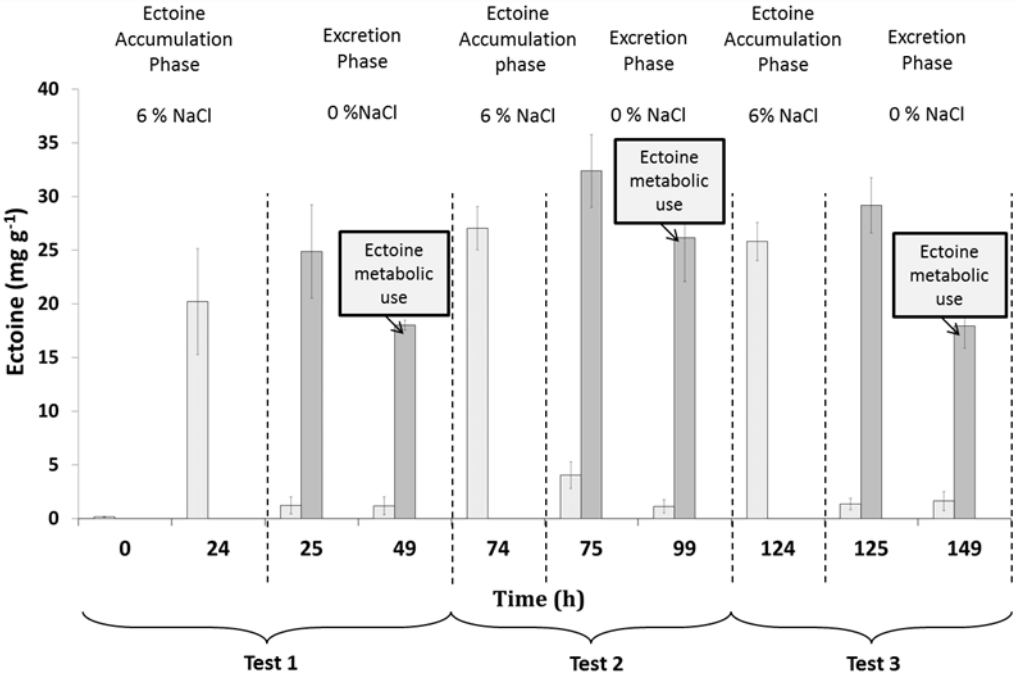
Pressure regulator
Air compressor
Mass flow controler
Methane 99.9 %

Fig.2


Ectoine Accumulation Phase
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Ectoine Accumulation phase
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Ectoine Accumulation Phase
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Ectoine metabolic use
Ectoine metabolic use
Ectoine metabolic use
Ectoine (mg g-1)
40
35
30
25
20
15
10
5
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24
25
49
74
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99
124
125
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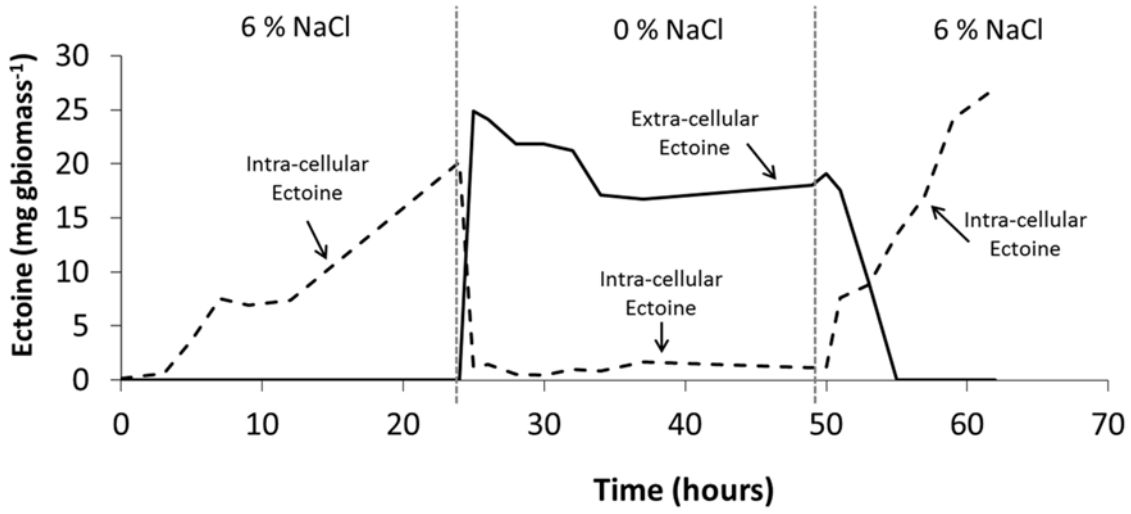
Time (h)
Test 1
Test 2
Test 3

Fig.3


6 % NaCl
0 % NaCl
6 % NaCl
Ectoine (mg gbiomass-1)
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25
20
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10
5
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Intra-cellular Ectoine
Extra-cellular Ectoine
Intra-cellular Ectoine
Intra-cellular Ectoine
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10
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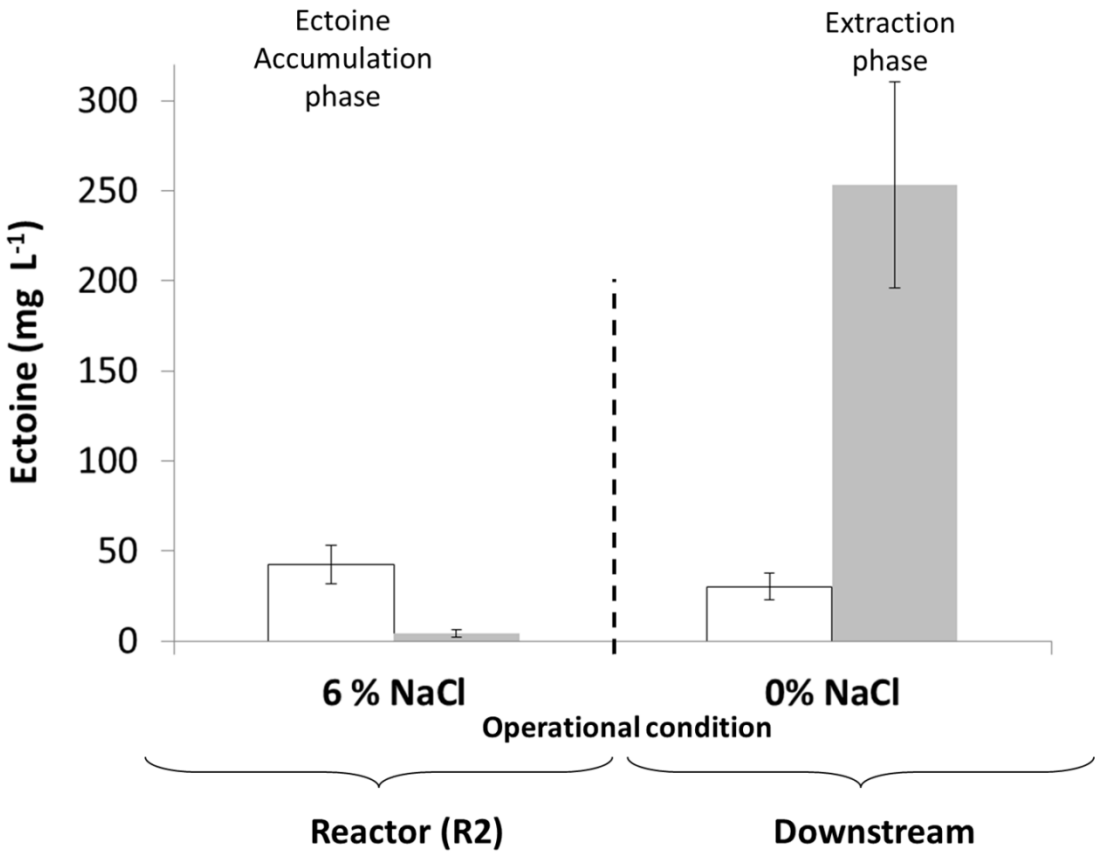
40
50
60
70
Time (hours)

Fig.4


Ectoine
Accumulation
phase
Extraction
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Ectoine (mg L-1)
6 % NaCl
0% NaCl
Operational condition
Reactor (R2)
Downstream

Supplementary Material

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