

Enhanced ectoine production by carbon dioxide capture: a step further towards circular economy

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Abstract:

Recycling of greenhouse gases to produce industrially valuable products has become one of the big pillars to achieve circularity. This study demonstrates for the first time the feasibility of producing ectoine with *Halomonas stevensii* with CO₂ as the added carbon source, and CO₂ and glucose. Initially, CO₂ alone was fed to continuous reactors, adding thiosulphate as the energy source. Maximum CO₂ elimination capacities of 24.2 mg CO₂ L⁻¹ h⁻¹ were obtained, and ectoine contents up to 7.3 % (g ectoine·g biomass⁻¹). To enhance ectoine production, CO₂ conversion coupled with discontinuous glucose addition was implemented. The amendment of 0.5 g L⁻¹ of glucose at the beginning of reactor operation enhanced CO₂ removal to 37.1 mg CO₂ L⁻¹ h⁻¹ and increased ectoine contents up to 22 %. Our results represent the proof of concept for a CO₂ biotransformation platform to produce ectoines, so far unexplored. This can foster the development of more sustainable microbial processes for the production of ectoines helping in the abatement of CO₂.

Keywords: sustainable economy, greenhouse gases, CO₂ biotransformation, halophiles, ectoine.

1. Introduction

Carbon dioxide (CO₂) emissions represent approximately 82 % of the total greenhouse gases (GHGs) released worldwide, and their share is expected to increase in the coming years (Metz et al., 2018; IPCC, 2018). To overcome this problem, current research and political initiatives focus on building a circular, resource-efficient and climate-resilient society (Anand et al., 2020). Recycling of GHGs for the production of industrially valuable products has become one of the big pillars to achieve circularity (Gabrielli et al., 2020).

Energy and industrial sectors are the main sources of anthropogenic CO₂ emissions with an annual discharge of 666 Mt of CO₂ to the atmosphere. CO₂ concentrations in flue gases are highly variable ranging from highly concentrated currents (50-70%) to lower emissions, such as natural gas-fired power plants (8-10% CO₂) or coal-fired boilers (12-14 % CO₂) (Song et al., 2004). This CO₂ can be used as feedstock for the production of materials, chemicals and fuels. In principle, the physico-chemical transformation of CO₂ is unattractive because of i) the high requirements of energy and chemicals, and ii) the technical and regulatory limitations for the logistics of CO₂ since these technologies usually require prior CO₂ capture, concentration, removal of impurities, and transportation to the application sites (Kumar et al., 2018). A viable, low energy-demanding option to transform CO₂ directly from flue gases, without prior accumulation or pre-treatment, entails the use of the catalytic activity of specialized microorganisms (Gabrielli et al., 2020; Khandelwal et al., 2021). CO₂ can be assimilated as cellular biomass and transformed into products with market value. So far, the use of CO₂ as a substrate to produce valuable chemicals relies mainly on the use of algae, which requires large implementation areas for photobioreactors (Bose et al., 2019). In the case of dark

CO₂ fixation, promising cell factories are being researched to produce bioplastics, biodiesels, single cell protein and extracellular polysaccharides (Amulya and Venkata Mohan, 2022; Garcia-Gonzalez et al., 2015; Kumar et al., 2019; Kumar and Thakur, 2020; Molitor et al., 2019; Yang et al., 2021). However, current processes apply to a restricted number of model microorganisms, such as acetogens, with a small portfolio of products, mainly alcohols and organic acids (Zeng, 2019). Thus, the expansion of both, the portfolio of valuable products synthesized from CO₂ and, the diversity of organisms used for CO₂ conversion is of great interest.

Among the secondary compounds produced by microorganisms, ectoines have one of the highest market prices with a retail value of 1000 € kg⁻¹ (Becker and Wittmann, 2020). Ectoines (ectoine and hydroxyectoine) are synthesized by halophilic bacteria to survive in salt-rich environments. They are effective stabilizers for enzymes, DNA-protein complexes, and nucleic acids (Liu et al., 2021), which makes of them a target product in the pharmaceutical industry (Becker and Wittmann, 2020). Despite their value and demand, ectoines are only commercialized by the company BITOP (Witten, Germany), which synthesizes them at the ton scale. In their process, BITOP uses bacteria from the genus *Halomonas*; glucose is supplied as sole carbon source, inflating production cost (Cantera et al., 2018; Kunte et al., 2014).

Bacteria of the genus *Halomonas* are the ectoine producers per excellence. They can grow at salinities up to 35% NaCl and are reported to produce from 150 to 358 mg of ectoine per gram of biomass (Chen et al., 2014, 2019; Zhang et al., 2009). Recent studies have shown that *Halomonas stevensii* can grow using thiosulfate as the energy donor without an extra apparent carbon source rather than CO₂ (Khandelwal et al., 2021; Mishra et al.,

2018, 2017), however, to date, CO₂ utilization for ectoines production has not been reported.

The removal of thiosulfate is important from an environmental point of view, due to the severe pollution problems that it causes if discharged without treatment (corrosion of the sewer pipes, eutrophication, silting hydrogen sulfide formation, etc.). The accumulation of thiosulfate in industrial wastewater from the chemical sector (petrochemical, metallurgical, photography processing, pharmaceutical, pigment, dye manufacturing units, etc.) increases every year. Hence, research is encouraged for the development of suitable treatment methods to reduce the concentration of thiosulfate in aqueous solution down to permissible limits (Ahmad et al. 2015).

Thus, this work explores for the first time the biotechnological potential of the genus *Halomonas* as a new platform for the enhanced production of ectoines with CO₂ incorporation and thiosulfate removal. CO₂ utilization by *H. stevensii* was tested in aerobic bioreactors adding thiosulfate as energy source. Besides, different salinities and thiosulfate concentrations were evaluated. We further studied the effect of adding low concentrations of glucose on CO₂ uptake and ectoines productivities.

2. Materials and Methods

2.1. Chemicals and mineral salt medium

The mineral salt medium (MSM) used for the growth of *Halomonas* was composed by (g L⁻¹): KNO₃ - 1, K₂HPO₄ - 1, MgSO₄·7H₂O - 0.2, CaCl₂·2H₂O - 0.02, NH₄Cl – 0.16 and trace elements (mg L⁻¹): (CuCl₂·5 H₂O - 0.1, FeSO₄·7 H₂O - 2, ZnSO₄ x 7 H₂O - 0.1, NiCl₂·6 H₂O - 0.02, CoCl₂ x 6 H₂O - 0.2, Na₂MoO₄ - 0.3, MnCl₂ x 4 H₂O - 0.03, H₃BO₃ - 0.03). MSM was autoclaved at 1.5 atm at 121°C for 20 min. Na₂S₂O₃ was added after

sterilization up to the different concentrations tested (30, 20 and 10 mM) from a 1 M sterile stock. The pH of the MSM was adjusted using 5 M NaOH stock solution after autoclavation. NaCl was added during MSM preparation at the concentrations tested in each experimental run.

2.2. Microorganisms

Different *Halomonas* strains (*H. alkalicola*, *H. campaniensis*, *H. elongata* and *H. stevensii*; supplementary materials, Table S1) were acquired from the DSMZ culture collection (Leibniz-Institut, Germany) and were grown in Bacto marine broth (Difco Medium 2216). An aliquot of 1 mL of each stock liquid culture was inoculated in 200 mL glass bottles containing 100 mL of MSM supplemented with 6 % NaCl and 10 mM Na₂S₂O₃. Bottles were closed using gas-tight butyl septa and aluminum caps. CO₂ was then injected to the headspace at an initial concentration of 20/80 (v/v) CO₂/air (CO₂ was added after removal of 20% of air to maintain atmospheric pressure). The cultures were incubated at 30 or 37 °C (supplementary materials, Table S1) under orbital agitation at 200 rpm. The cultures were transferred three consecutive times to the aforementioned supplemented medium under an atmosphere of 20/80 (v/v) CO₂/air to remove any remanent carbon sources. As positive controls, these strains were also transferred to glass bottles containing 100 mL of MSM supplemented with 6 % NaCl and 1 g L⁻¹ glucose.

2.3 Analytical procedures

High performance liquid chromatography (HPLC) was used to measure extra and intra cellular organic metabolites, ectoine, hydroxyectoine and the electron donor and acceptors, thiosulphate, sulphate, sulphite and sulphur. In all cases, the detection and quantification limits (DL and QL) were estimated using the signal-to-noise ratio

(comparison between signals from samples with known low concentrations of analyte with those of blank samples).

The intra-cellular ectoine and hydroxyectoine contained in 2 mL of cultivation broth was extracted in duplicate, following the protocol described by Cantera et al. (2016). The concentration of ectoine was measured by HPLC-UV in a HPLC LC_2030_C_Plus_2_ELSD (Shimadzu, Japan) at 210 nm and 35 °C using a Spherisorb Amino (NH₂) Column, 80Å, 3 µm, 4.6 mm X 150 mm (Waters, USA). The mobile phase consisted in acetonitrile/H₂O 75/25 (%) at a flow rate of 1 mL min⁻¹. Ectoine, hydroxyectoine and betaine 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 %] and hydroxyectoine [(4S,5S)-5-Hydroxy-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid purity 95%, (Sigma Aldrich, Germany). The specific intra-cellular ectoine content (%= g ectoine g biomass⁻¹ ·100) was calculated using the corresponding dry biomass concentration (g L⁻¹).

Thiosulphate, sulphate and sulphite were monitored in all the reactors throughout operation with anion exchange chromatography. Sulphate was measured on a Dionex ICS-2100 (Dionex, USA) equipped with a Dionex IonPac AS16 column (Dionex, USA) and sulphite and thiosulphate were measured on a Dionex ICS-2100 (Dionex, USA) equipped with a Dionex IonPac AS17 column (Dionex, USA). One ml of media was centrifuged at 13000 g for 10 min. 30 µL of the supernatant was transferred to HPLC vials containing 970 µL of milli-Q water. 5 % of pure methanol was added to the mixture with the aim of preserving the samples until analysis. Sulphur was extracted from 0.5 ml samples with chloroform in a shaking bath at 650 rpm, 25°C for 1 h. Then, the upper phase was discarded, and 750 µL of methanol were added to 250 µL of sample. The

samples were filtered and measured on an Accela High Speed LC (Agilent, USA) equipped with a Li-Chrospher 100, RP C₁₈ column (Agilent, USA). The mobile phase consisted on methanol 100 and the flow rate was 1 mL min⁻¹. The peaks were detected using UV absorption wavelength of 263 nm. Concentrations of organic acids and monosaccharides were determined on a Shimadzu LC2030c (Shimadzu, Japan) equipped with a Shodex SH1821 column (Shodex, Japan) and a differential refractive index detector Shimadzu RID-20A (Shimadzu, Japan) operated at 45°C, with 5 mM H₂SO₄ as eluent at a flow rate of 1 mL min⁻¹. Both refractive index (RI) and ultraviolet (UV) detectors were used. The samples were prepared adding 800 µL of sample and 200 µL of the internal standard (0.1 mM of crotonic acid).

CO₂ was measured in a Shimadzu Gas chromatograph with a Thermal Conductivity Detector (GC-TCD 2014; Shimadzu, Japan) equipped with a CP Poraplot Q column, CP7554, (25 m × 0.53 µm × 20 µm). The oven, injector and detector temperatures were maintained at 45, 150 and 200 °C, respectively. Helium was used as carrier gas at 13.7 mL min⁻¹. The CO₂ elimination capacity (CO₂-EC) was calculated using Eq1.

$CO_2 - EC = \frac{Q \times ([CO_2]_{in} - [CO_2]_{out})}{VR}$; where Q= inlet gas flow, [CO₂]_{in}= CO₂ inlet concentration, [CO₂]_{out}= CO₂ outlet concentration, VR= Reactor volume.

Optical absorbance measurements at 600 nm (OD₆₀₀) were conducted using a UV/Vis spectrophotometer (Shimadzu, Japan). Dry biomass concentration was measured as total suspended solids according to Standard Methods. pH was determined using pH-probes associated to the DASGIP Bioblock controller. The concentrations of dissolved inorganic carbon (DIC) were measured with a Shimadzu TOC-VCSH analyzer (Japan) equipped with a TNM-1 chemiluminescence module.

2.4 CO₂ uptake by *Halomonas stevensii*- Test 1

Test 1 was performed to assess the growth of *H. stevensii* in batch bottles where CO₂ was the only carbon source provided. In Test 1, 1 mL of *H. stevensii* culture acclimatized to CO₂ (section 2.2) was transferred to triplicate 120 ml serum bottles filled with 50 mL of MSM supplemented with 10 mM Na₂S₂O₃. Prior inoculation, 12 ml of air were removed from the bottles and the same amount of CO₂ was added (atmospheric pressure). Abiotic negative controls, prepared in the same way as test bottles but without inoculum addition were also set-up. Different pH (5.5, 6.5, 7.2, 8, 9), were tested in batch as described above to select the best pH for CO₂ abatement.

2.5 Ectoine production enhanced by CO₂ assimilation – Test 2

Test 2 was carried out as first proof of concept of ectoine production associated with CO₂ elimination in continuous bioreactors. For this, four 1-L Eppendorf stirred-tank reactors (STRs) from the DASGIP Bioblock system (Eppendorf, Hamburg, Germany) were used in combination with the DASGIP TC4SC4B module for temperature and agitation control and with the DASGIP MX4/4 gas mixing system for gas mass flow control. The STRs were filled with 950 mL of 6 % NaCl MSM containing 20 mM of Na₂S₂O₃. A 0.016 ± 0.005 L min⁻¹ CO₂-laden air stream containing 360.8 ± 18 mg CO₂ L (≈ 20 %; CO₂ load of 345 mg L h⁻¹) was fed to the STRs via porous stainless steel diffusers located at the bottom of the reactors. This gas stream was obtained by mixing a continuous CO₂ stream (99.9% purity) with air in a mixing chamber connected to the mass flow controller of the DASGIP MX4/4 and regulated by the DASGIP® WRM rotameter gassing station (Eppendorf, Hamburg, Germany). The gas empty bed residence time (EBRT) was set at 60 minutes. Evaporation losses were avoided using the DASGIP cooling water distribution unit placed on each exhaust condenser. Prior to inoculation, an abiotic test

with sterile medium was performed for 5 days at the above described operational conditions to assess any potential removal of CO₂ by chemical reactions in the experimental set-up. Afterwards, 50 mL of *H. stevensii* culture were used to inoculate the reactors. The reactors were operated for 45 days at 30°C with an agitation of 400 rpm. The first 6 days of reactor run were carried out in semicontinuous mode (gas was fed in continuous while the liquid dilution rate was 0) to allow for biomass retention. During semicontinuous mode, 5 mL were daily removed from the bioreactors to determine OD₆₀₀, ectoine and hydroxyectoine. From day 7, 50 mL of culture broth were replaced by fresh MSM every day to maintain optimal nutrient and thiosulphate concentrations and to prevent the accumulation of potential inhibitory metabolites, such as sulfite resulting in a final HRT of 20 days. The withdrawn cultivation broth (50 mL) was used for the determination of OD₆₀₀ and TSS, sulphur species, ectoine, and hydroxyectoine.

2.6 Influence of salinity in ectoine contents and CO₂ abatement – Test 3

A third test, Test 3, was carried out with the aim of optimizing ectoine production using different salinities. To this aim, four different salt concentrations were tested in STRs, namely: STR_6 (6% NaCl), STR_9 (9% NaCl), STR_12 (12% NaCl), STR_20 (20% NaCl). The STRs were filled with 900 mL of MSM supplemented with 30 mM of sodium thiosulphate (Fig.1). A $0.018 \pm 0.001 \text{ L min}^{-1}$ CO₂-laden air stream containing $360.5 \pm 20.2 \text{ mg CO}_2 \text{ L}^{-1}$ ($\approx 20 \%$), corresponding to a CO₂ load of $373.2 \pm 25.7 \text{ mg CO}_2 \text{ L}^{-1} \text{ h}^{-1}$, was fed to the STRs via porous stainless steel diffusers located at the bottom of the reactors. The EBRT was set at 60 min. Prior to inoculation, an abiotic test with MSM was performed for 5 days. Afterwards, 100 mL of an exponentially grown culture of *H. stevensii* were added. The reactors were operated at pH 7.4 ± 0.3 , 30 °C with an agitation of 400 rpm. Steady state was considered when the CO₂ elimination capacity (CO₂-EC)

deviated <10% from the mean. 5 mL of culture broth were removed from the bioreactors on days 1, 3 and 5 to determine OD₆₀₀ and ectoine and hydroxyectoine. From day 6 on, 100 mL of culture broth were replaced by fresh medium every day to prevent the accumulation of sulphur and to avoid the limitation of thiosulphate (HRT = 10 days). This broth was used for the determination of OD₆₀₀, TSS, DIC, organic acids, sulphate, sulphite, sulphur and thiosulphate, ectoine and hydroxyectoine. Gas samples for CO₂ analysis were daily taken using the sampling ports located at the inlet and outlet of the bioreactors using 100 µL gas-tight syringes (HAMILTON, Australia).

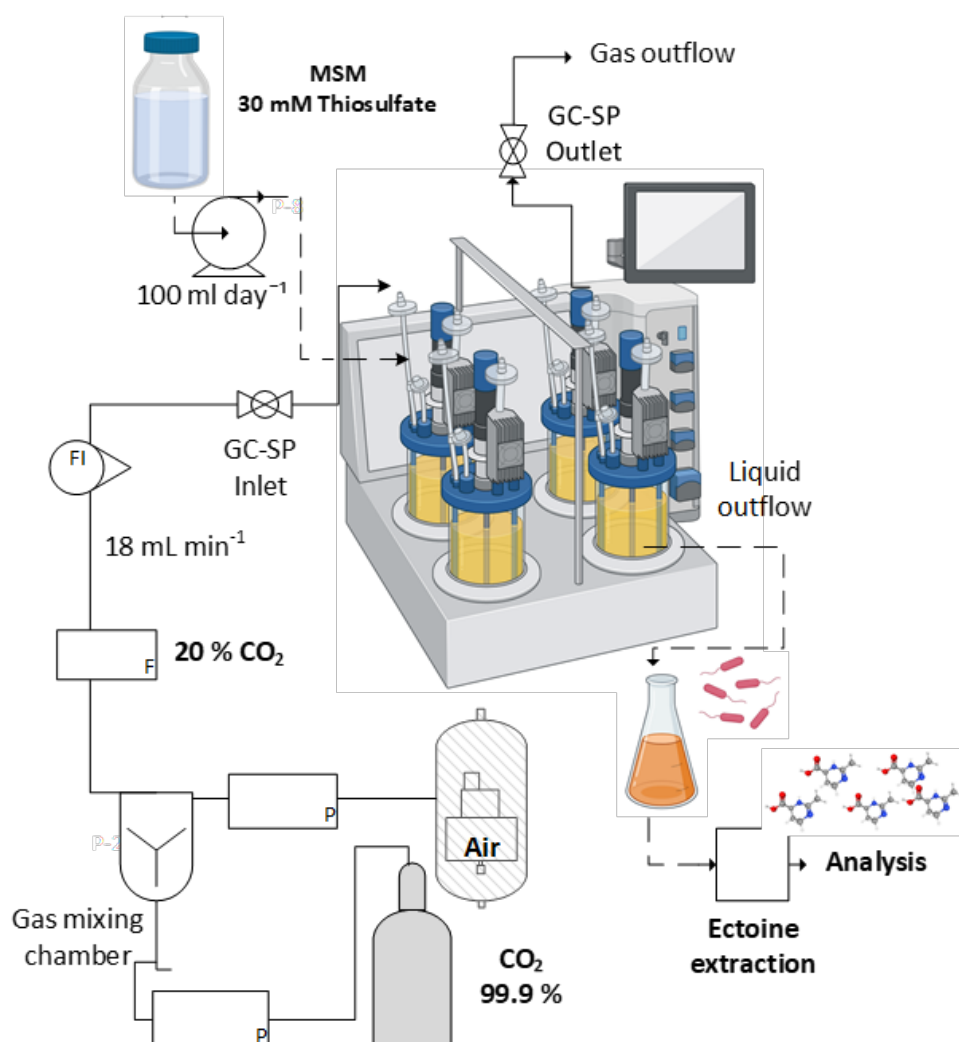


Fig. 1: Schematic diagram of the experimental setup: Pressure meter (P), Flow mass controller (F), Rotameter (FI), Mineral salt media (MSM) storage tank, Gas streams (continuous line), Liquid streams (discontinuous line), Gas chromatograph sampling port (GC-SP).

2.7 Influence of glucose addition on CO₂ elimination and ectoine production – Test 4

The last test, Test 4, attempted to study the influence of the discontinuous addition of 0.5 g L⁻¹ glucose in ectoine production and CO₂ abatement by *H. stevensii*. The selection of the glucose concentration was based on the external carbon requirements for other mixotrophic bacteria (Smith et al., 2015). Two STR_S bioreactors containing 950 ml of MSM supplemented with 30 mM of sodium thiosulphate were operated at 6 and 12% NaCl. The reactors were inoculated with 50 mL of *H. stevensii*-containing liquid broth from reactors described in 2.5. According to their salinity the reactors were named: STR6_GLC (6% NaCl) and STR12_GLC (12% NaCl). 10 mL of the culture broth were withdrawn from the bioreactors daily in the first 5 days to determine OD₆₀₀, TSS, sulphur species and ectoine and hydroxyectoine. From day 5, 100 mL of culture broth were replaced by fresh medium every day (HRT 10 days). These 100 mL of cultivation broth were used for the determination of OD₆₀₀, TSS, IC, sulphate, sulphite and thiosulphate, as well as ectoine and hydroxyectoine. Gas samples for CO₂ analysis were daily taken from the sampling ports located at the inlet and outlet of the bioreactors using 100 µL gas-tight syringes (HAMILTON, Australia).

2.8 Purity check of *H. stevensii* cultures

All the bioreactors operated in this work were regularly checked for potential microbial contamination. Routine purity check consisted in the collection of liquid broth, isolation of total DNA, and subsequent sequencing of the 16S rRNA gene (Sanger sequencing). 16S rRNA genes were amplified by PCR using a Taq DNA polymerase kit (Invitrogen, Carlsbad, CA, USA) with the primer set 27-F (5'- AGAGTTTGATCCTGGCTCAG-3)

and 1492-R (5- GYTACCTTGTTACGACTT- 3). PCR program was as follows: 95 °C for 10 min; 35 cycles at 95 °C for 30 s, 55 °C for 40 s, and 72 °C for 90 s; and a final elongation cycle at 72 °C for 10 min. PCR products were purified using the Zymo DNA Clean & Concentrator kit (Zymo Research, USA) and sequenced by Macrogen Europe B.V. (Netherlands). The 16S rRNA gene sequences were analysed using the DNA baser software (version 4.20.0. Heracle BioSoft SRL, Pitesti, Romania). The parameters set to determine a high-quality sequence were: a) average quality before trimming ≥ 42 ; b) average quality after trimming ≥ 50 . All the sequences obtained belonged to the specie *H. stevensii* (similarities 99-100%, depending upon quality of the sequence). The accession numbers in GenBank are: MW888858-MW889008.

In addition, at the end of bioreactor operation, 16S rRNA gene amplicon sequencing (Illumina Mi-seq sequencing) was carried out. Primer set S-D-Bact-0341-b-S-17/S-D-Bact-0785-a- A-21, targeting V3 and V4 regions of the 16S rRNA gene, was used (Pascual et al. 2020). The 16S rRNA gene sequences were processed and quality filtered using Mothur v1.44.3 following the Mother SOP (https://www.mothur.org/wiki/MiSeq_SOP) (Schloss, 2020). Sequences were then clustered at 97% identity threshold into Operational Taxonomic Units (OTUs) using the SILVA 16S rRNA gene reference database (Version: 138.1) (Quast et al., 2013). Microbial diversity was analyzed using R version 1.4.1 (Dia et al., 2017) and heatmaps plotted using the package *pheatmap* (R package version 1.0.12) (Supplementary fig. S1 and S2). All sequences obtained (deposited at GenBank as bioproject: PRJNA689702) were >90 % related to *Halomonas*, ruling out a possible microbial contamination of the bioreactors.

2.9 Data analysis

Statistical analyses were done using SPSS 20.0 (IBM, USA) according to the procedure described by Cantera et al. (2021). Analytical results are given as the average of biological replicas \pm standard deviation and technical replicas \pm standard deviation depending on the experiment.

3. Results and Discussion

3.1 CO₂ elimination by *Halomonas stevensii*

Test 1 consisted on batch cultivations to test if members of the ectoine producing *Halomonas* genus were able to eliminate CO₂ with thiosulfate as added substrate. *H. alkalicola*, *H. campaniensis* and *H. elongata* did not show activity or growth in contrast with the positive controls with glucose. However, *H. stevensii* was able to completely use the supplied CO₂ (79.8 ± 7.7 mg CO₂ L⁻¹ in the headspace at initial pH 6.7), in association with an increase in biomass concentration of 35.4 ± 1.9 mg biomass L⁻¹. This result showed that ~ 50 % of the carbon provided to *H. stevensii* was assimilated as biomass (Fig. 2A), a somehow high value which triggered us to hypothesise that *H. stevensii* could also be very efficiently using small amounts of organics transferred from previous cultures resulting in mixotrophic growth. Negative controls (uninoculated) did not show CO₂ depletion, thus the removal of CO₂ from the headspace in *H. stevensii* cultures was linked to biological activity.

The pH value in the incubations increased to 7.9 due to CO₂ removal and thiosulfate oxidation. From the range of pH tested (5.5, 6.5, 7.2, 8.3, 9), pH 7.2 combined optimal growth of *H. stevensii* with accurate biomass quantification avoiding errors due to the turbidity caused by highly carbonated liquids at increased pH. Moreover, it allowed to

quantify more precisely CO₂ elimination in the headspace. At pH 8.3 and 9, CO₂ was sequestered in the liquid phase as carbonate and could not be detected in the headspace. Previously, Mishra et al. (2017) had already observed complete CO₂ depletion (of 10% v/v CO₂ in the headspace) by *H. stevensii* with thiosulphate as electron donor (100 mM) at pH 10 in batch experiments. However, the biomass yields obtained in their experiments were much higher than the ones recorded here (860 ± 0.02 mg biomass L⁻¹) (Mishra et al., 2018, 2017). These high biomass yields could be due to the higher available CO₂ in the liquid broth at the higher pH used or the presence of an unknown carbon source in the cultures that could be growing in mixotrophy.

3.2 Ectoine production from thiosulphate and CO₂

Sterile bioreactors continuously fed with CO₂ to ensure enough carbon available were operated to measure ectoine and hydroxyectoine accumulation in Test 2. Reactors operated abiotically for 5 days were used as control. In the abiotic reactors the OD₆₀₀ and pH did not vary (0.005 ± 0.003 and 7.45 ± 0.05 respectively) and thiosulfate and sulfate concentrations were constant (15.1 ± 0.6 mM S₂O₃²⁻ and 0.8 ± 0.1 mM SO₄²⁻). The biomass concentration in the bioreactors increased to values of 92.5 ± 4.8 mg biomass L⁻¹ (Fig. 2B; cell counting and microscope observations in supplementary materials 1, Fig. S3 and Fig. S4). Thiosulphate was consumed at a rate of 0.5 ± 0.03 mM h⁻¹ (average 1.0 ± 0.1 mM S₂O₃²⁻ detected in the media throughout operation) with the production of sulphate (12.3 ± 0.5 mM SO₄²⁻) and sulphur ~ 3 -6 mM S⁰ at the end of the operation (accurate quantification of elemental sulphur was not possible due to its precipitation and adherence to the reactor walls). Sulphite was not detected in the medium probably due to its fast oxidation to sulphate in the presence of oxygen.

Ectoine was detected in all the bioreactors with maximum contents during cell exponential growth ($4.8 \pm 0.6\%$ on dry weight basis). Intra-cellular ectoine concentration usually peaks in the mid-exponential growth phase, due to the initial hyperosmotic shock, and decreases afterwards during the growth-retardation phase being re-assimilated by cell metabolism (Czech et al., 2018). During steady state, ectoine contents obtained were $3.3 \pm 0.8\%$ at 6% of NaCl (Fig. 2B). Ectoine contents obtained were much lower than those usually reported for glucose-grown *Halomonas* species, typically in the range of 15-35% (Becker and Wittmann, 2020). In addition to glucose being a better carbon source, most of previous studies used a high-salinity medium (NaCl concentrations from 15-20%), which favours ectoine production (Becker and Wittmann, 2020; Pastor et al., 2010).

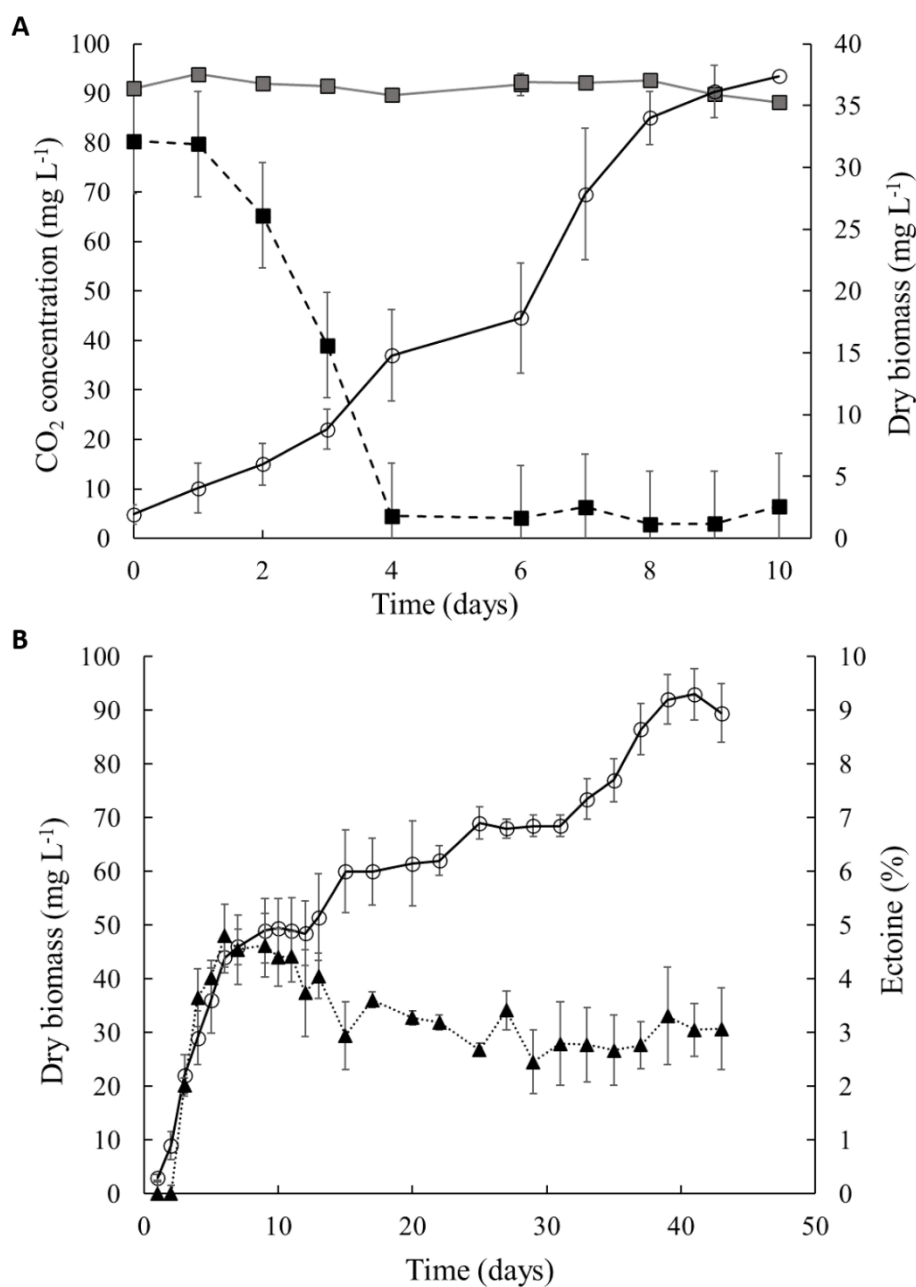


Fig.2. A) Growth and CO₂ abatement by *H. stevensii* in batch bottles, Test 1, using 10 mM of Na₂S₂O₃ as energy source and pH of 7 (data shown as mean of triplicates (\pm) standard deviation (mean \pm SD)). B) Growth of *H. stevensii* and ectoine production in four independent bioreactors in Test 2. 20 mM of Na₂S₂O₃ were used as energy source (data shown as mean of quadruplicates (\pm) standard deviation (mean \pm SD)). CO₂ was the only carbon source supplied in both batch bottles and bioreactor experiments. Grey line: abiotic control, ○-black line: biomass concentration, ■-dashed line: CO₂ concentration, ▲-dotted line: ectoine accumulation.

3.3 Influence of salinity on CO₂ bioconversion

Higher salinities (6-20% NaCl) and sodium thiosulphate concentrations (30 mM) were used in a follow up experiment, Test 3. No significant CO₂ or thiosulphate degradation occurred along the abiotic removal test after stabilization for 5 days, as shown by the negligible difference (<5%) between inlet and outlet CO₂ gas and thiosulfate concentrations in the four bioreactors. A constant pH of 7.4 ± 0.2 was recorded in the four bioreactors during the abiotic test (Table 1).

Process operation at the four different NaCl concentrations was characterized by a steady performance on CO₂ removal from day ~15 onwards. The DIC results showed a decrease of 17%, 19% and 12% of the total dissolved inorganic carbon in STR_6, STR_9 and STR_12, respectively (Table 1). This confirmed that carbonate and bicarbonate were being removed by the bacterial population. In the reactor operated at 20% NaCl decrease in DIC was almost negligible. The EC-CO₂ obtained in STR_6 ($23.9 \pm 2.9 \text{ mg L}^{-1} \text{ h}^{-1}$) was statistically equal to the one recorded in STR_9 ($22.4 \pm 2.7 \text{ mg L}^{-1} \text{ h}^{-1}$). At 12% NaCl, the bacterial performance in CO₂ removal was significantly reduced ($17.5 \pm 1.9 \text{ mg L}^{-1} \text{ h}^{-1}$) and at 20% NaCl, the high salinity concentration probably hindered bacterial growth and the EC-CO₂ was negligible (Table 1, Fig.3).

Table 1: Average steady values obtained in Test 3 and 4 operational runs											
Reactor	NaCl (g L ⁻¹)	pH	EC-CO ₂ (mg L ⁻¹ h ⁻¹)	DIC (mg L ⁻¹)	Biomass (mg L ⁻¹)	Biomass (mg L ⁻¹ d ⁻¹)	S ₂ O ₃ ²⁻ (mM)	SO ₄ ²⁻ (mM)	S ⁰ (mM)	Ectoine (%)	Hydroxyectoine (%)
Test 3: Bioreactors operated with CO ₂ at different salinities											
Abiotic	All	7.4 ± 0.2	N.D (p<0.05)	1682 ± 52	N.D.	N.D.	27.1 ± 0.9	0.8 ± 0.01	~0.7	N.D.	N.D.
STR_6	60	7.2 ± 0.1	23.9 ± 2.9	1409 ± 66	236.5 ± 16.4	23.8 ± 1.4	3.1 ± 1.5	15.5 ± 1.0	~6.5	2.6 ± 0.4	0.02 ± 0.01
STR_9	90	7.3 ± 0.2	22.4 ± 2.7	1367 ± 22	239.2 ± 37.4	24.2 ± 1.1	2.8 ± 0.1	14.0 ± 0.2	~7.0	4.2 ± 0.2	0.3 ± 0.04
STR_12	120	7.4 ± 0.2	17.5 ± 1.9	1486 ± 24	179.6 ± 11.5	17.9 ± 1.2	5.9 ± 2.1	10.5 ± 3.3	~5.5	7.3 ± 0.7	0.5 ± 0.05
STR_20	200	7.5 ± 0.1	1.9 ± 4.8	1622 ± 34	N.D	N.D.	18.3 ± 4.9	4.8 ± 0.8	~3.5	0.8 ± 0.6	N.D.
Test 4: Bioreactors operated in mixotrophy with CO ₂ and 0.5 g L ⁻¹ glucose											
Abiotic	60/120	7.3 ± 0.1	N.D (p<0.05)	1698 ± 44	N.D.	N.R.	25.6 ± 0.8	N.D	~1.0	N.D	N.D
STR6_GLC ^a	60	7.2 ± 0.2	37.1	N.R.	396.1	N.R.	3.7	14.9	N.R.	15.1	1.7
STR12_GLC ^a	120	7.3 ± 0.2	32.9	N.R.	270.9	N.R.	3.2	14.5	N.R.	22.9	0.08
STR6_CO ₂ ^b	60	7.4 ± 0.2	24.4 ± 0.7	1315 ± 30	226.6 ± 8.7	22.7 ± 0.8	3.6 ± 0.9	15.0 ± 2.8	~5.2	3.2 ± 0.2	N.D.
STR12_CO ₂ ^b	120	7.4 ± 0.1	18.4 ± 1.5	1321 ± 42	169.7 ± 9.8	16.7 ± 0.5	3.8 ± 1.0	15.5 ± 3.1	~4.4	7.8 ± 1.5	0.7 ± 0.2

All: All the salinities tested, 6, 9, 12 and 20 % NaCl. N.D: Not detected; N.R: Not recorded; a: maximum values during glucose consumption; b: average values of the steady state when glucose had been depleted.

1 These results were aligned with the biomass concentration achieved (Table 1, Fig.3). The
2 biomass obtained was statistically higher in STR_6 and STR_9 (236.5 ± 16.4 and 239.2
3 ± 37.4 mg L⁻¹, respectively). In fact, the biomass obtained at 6% NaCl was higher than
4 the one found in the previous operation (92.5 ± 4.8 mg L⁻¹). We correlated this result with
5 the higher amounts of S₂O₃ available for the bacteria due to the higher dilution rates and
6 with the potential removal of toxic sulphur species. In STR_12, the biomass concentration
7 under steady state was significantly lower (179.6 ± 11.5 mg L⁻¹). Although dry biomass
8 values were very low (26.8 ± 6.3 mg biomass L⁻¹) in STR_20, high OD₆₀₀ were recorded.
9 Microscope observations corroborated that there were almost no floating cells but orange
10 precipitates that were accumulating in this bioreactor, probably caused by the high salt
11 concentrations. According to the carbon balance analyses, the biomass hourly produced
12 in steady state (Table 1) showed that around 10% of the carbon from CO₂ went to biomass
13 in STR_6, STR_9 and STR_12, around 0.5% went to the production of organic acids
14 (supplementary materials, Table S2) that were excreted to the medium, and the rest
15 remained as bicarbonate/carbonate in the liquid phase.

16 Upon steady state operation of inoculated bioreactors, thiosulphate concentration dropped
17 by 88.5% in STR_6, 93.3% in STR_9 and 79.0% in STR_12 concomitant to an increase
18 in the sulphate and sulphur concentrations (Table 1). These results corroborated that *H.*
19 *stevensii* was using thiosulphate as an energy source. In the case of STR_20, the
20 thiosulfate concentrations found in steady state were only 32.0% lower than the ones
21 added to the reactors. This fact demonstrates again that bacterial activity was hindered by
22 the high salinity of the medium.

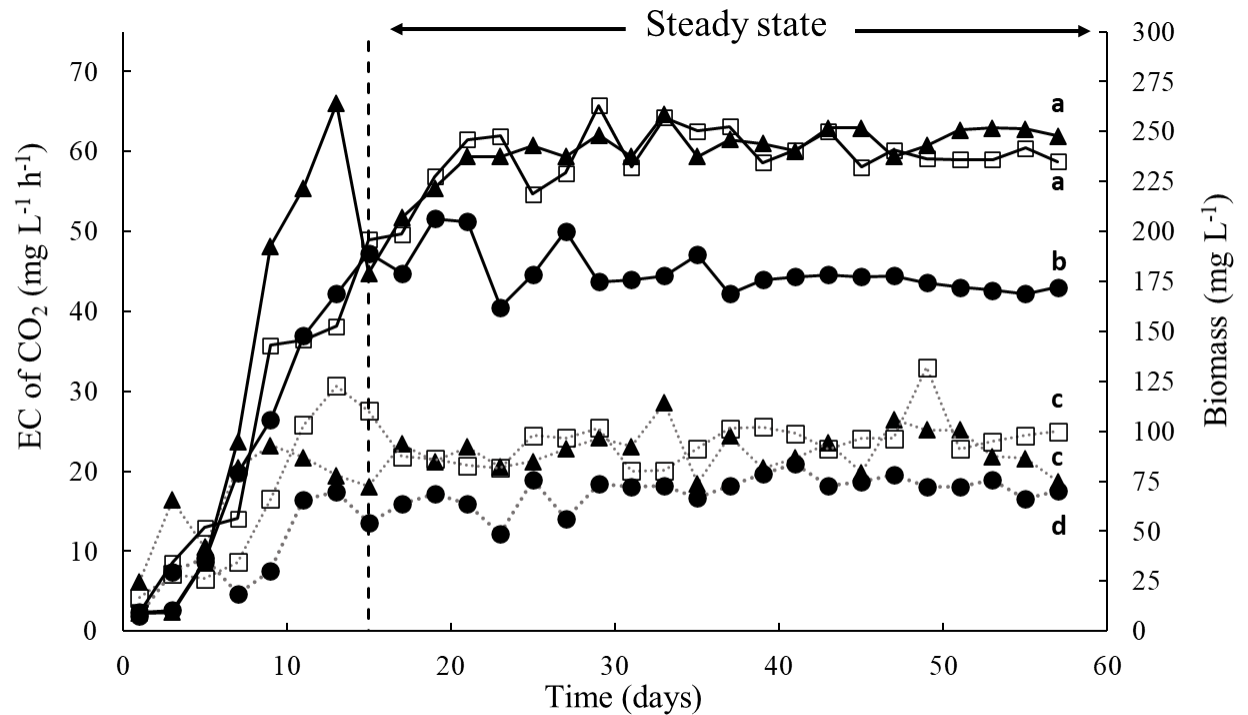


Fig.3. Represents the results of Test 3. Time course of the EC-CO₂ at: 6 % NaCl (□-dotted line), 9 % NaCl, (▲-dotted line) and 12% NaCl (●-dotted line). Time course of the biomass concentration at: 6% NaCl (□-black line, 9% NaCl(▲-black line) and 12% NaCl (●-black line). Different letters at the end of each line show significant difference between average values of each condition at $p < 0.05$.

Mishra et al. (2018) operated reactors in continuous with *H. stevensii* (15% of CO₂ v/v and 100 mM thiosulphate) and obtained biomass concentrations much higher than the ones detected in this study (4.6 g biomass L⁻¹). They assumed that thiosulphate was completely oxidized to sulphate producing enough energy to assimilate 100% of the CO₂ added, however sulphur species were not measured in their study (Mishra et al., 2018). Members of the *Halomonas* genus have been identified as able to oxidize thiosulphate in similar rates than sulphur oxidizing bacteria (Vavourakis et al., 2019). However, this oxidation of thiosulphate was partial and the product obtained was tetrathionate that in neutral conditions decomposes to sulphate and sulphur (Sorokin, 2003). In our study, it

was observed that in the presence of *H. stevensii*, sulphate and sulphur accumulated in the reactors during operation, compounds that were not found in the abiotic reactor. This result suggests that thiosulphate is not completely oxidized to sulphate, if not oxidized to tetrathionate that chemically decomposes in sulphur and sulphate, thus, contradicting what was hypothesized by Mishra et al. (2017, 2018). The low biomass yield might be related to the low energy provided by the partial oxidation of thiosulphate to tetrathionate. It has been previously observed in several *Halomonas* species that thiosulphate oxidation stimulates CO₂ assimilation (Sorokin et al, 2013), however, the capacity of *Halomonas* to thrive on the oxidation of thiosulphate to tetrathionate has been questioned due to the low energy production of this reaction (8 times lower than the complete conversion to sulphate) (Sorokin, 2003). In this regard, it seems plausible that *Halomonas* species require mixotrophy to efficiently assimilate CO₂ and produce high amounts of biomass.

3.4 Influence of salinity on ectoine production

During steady state, intra and extra cellular ectoine and hydroxyectoine content were comparatively evaluated at the different salinities tested in Test 3 (Fig. 4). The highest intra-cellular ectoine concentration was found in STR_12 where the cells accumulated 7.3 % of intracellular ectoine (on dry weight basis). In STR_9 and STR_6, the concentrations of intracellular ectoine were significantly lower with values of 4.2% and 2.6%, respectively. In STR_20, the concentrations of ectoine was almost negligible due to the absence of biomass in the samples. Although some ectoine producers excrete ectoine to the medium (Cantera et al., 2017a; Chen et al., 2014), in this experiment extra-cellular ectoine was only detected at very low concentrations in the reactor operated at 20% of NaCl, which could be due to cell lysis. The bacteria probably assimilated external ectoines resulting from cell apoptosis or membrane leaks to keep them as intra-cellular

ectoines and to use them as an efficient energy source for their own cell anabolism. This is in agreement with other studies reporting the use of extra-cellular ectoine in energy-yielding reactions under energy stress scenarios (Cantera et al., 2016; Kalyuzhnaya et al., 2008). In the case of hydroxyectoine, the concentrations found were almost negligible at 6 and 20% NaCl. Some hydroxyectoine was detected at 9 and 12% NaCl (0.3% and 0.5%).

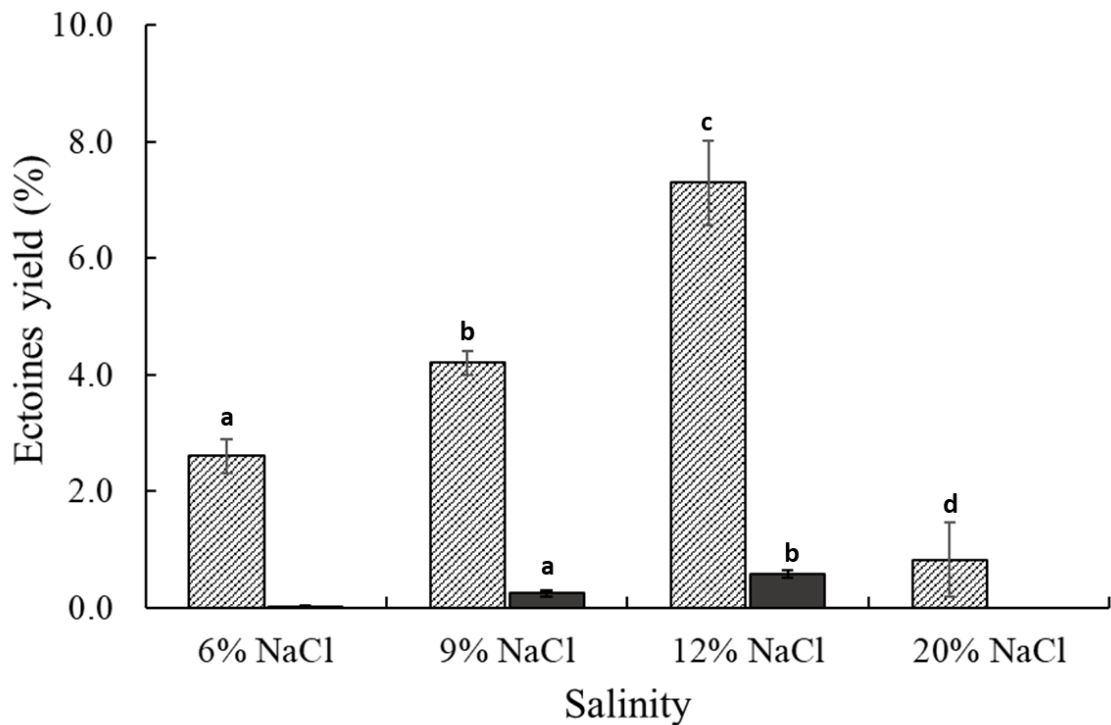


Fig.4. Influence of the concentration of NaCl on the steady state contents obtained of intra-cellular ectoine (dashed column) and hydroxyectoine (grey column) in Test 3. Vertical lines represent standard deviations from replicates. Columns intra-groups with different letters were significantly different at $p < 0.05$.

As expected higher ectoin contents were found at high salinities, except for 20% NaCl where the biomass was not able to grow. The contents of ectoine obtained are comparable

to those found with alkaliphilic methanotrophs using CH₄ as the sole carbon source (7-11%) (Cantera et al., 2017b, 2016). However, these values are very low in comparison with others obtained by *Halomonas* species growing in rich medium. The industrially implemented strain, *H. elongata* accumulates between 12 to 20% of intracellular ectoine and 2.0 to 3.5% of hydroxyectoine at salinities of 15-20% NaCl using glucose or glutamate as the main substrate (Becker and Wittmann, 2020; Liu et al., 2021; Pastor et al., 2010; Sauer and Galinski, 1998). These results showed that the bacteria were able to survive and maintain minimum cell metabolism with thiosulphate and CO₂, however, there was a clear limitation caused by the low energy supply during the oxidation of thiosulphate to tetrathionate. It has been observed in some *Halomonas* strains that CO₂ assimilation is increased in the presence of organic substrates (Sorokin, 2003). Therefore, a good strategy to increase ectoine production and CO₂ elimination while diminishing the costs of this biotechnology could be the discontinuous addition of low concentrations of more energetic organic sources, such as glucose, acetate or alcohols (Corti Monzón et al., 2018; Hobmeier et al., 2020).

3.5. Influence of glucose addition on ectoine production and CO₂ bioconversion

Two bioreactors were operated as described in section 3.4, but supplementing 0.5 g L⁻¹ of glucose at the beginning of the run in Test 4.

At 6% NaCl (STR6_GLC), in the first 72 hours, glucose was completely depleted (Table 1, Fig. 5). The biomass reached values of 351.2 mg biomass L⁻¹. With 0.5 g L⁻¹ of glucose, the bacteria could produce around 187.9 mg biomass L⁻¹ (considering a 50% flow of the carbon to biomass), however, this growth was connected to the elimination of CO₂ and an EC-CO₂ of 32.9 ± 1.9 mg CO₂ L⁻¹ h⁻¹ was recorded (202.8 mg biomass L⁻¹ day would be the potential biomass from the total CO₂ consumed considering that 50% of the carbon

is diverted to metabolism). After 24 hours (day 4) the biomass achieved values of 396.1 mg biomass L⁻¹ and an EC-CO₂ 37.1 ± 2.9 mg CO₂ L⁻¹ h⁻¹ probably using the energy of thiosulphate and polyhydroxyalkanoate and ectoine catabolism. Once glucose was completely depleted (STR6_CO₂) the biomass and the EC-CO₂ dropped and stabilized in values similar to those obtained in STR_6 with CO₂ as the only carbon source provided.

At 12% NaCl (STR12_GLC), the lag phase was longer and biomass content lower, i.e. 258.2 mg biomass L⁻¹ after the 96 hours necessary to completely deplete glucose (Table 1, Fig.5). This is possibly explained by the higher energy necessary to produce ectoine and to cope with salinity stress levels (Kunte et al., 2014; Pastor et al., 2010). An EC-CO₂ of 27.8 ± 0.8 mg CO₂ L⁻¹ h⁻¹ was observed. On day 6 the biomass had achieved the highest values at 12% (270.9 mg biomass L⁻¹) and an EC-CO₂ of 32.9 ± 1.4 mg CO₂ L⁻¹ h⁻¹ mg.

In STR12_CO₂, the biomass and the EC-CO₂ dropped and stabilized in values similar to the ones recorded in STR_12.

Thiosulphate and sulphate values recorded had similar concentrations during operation, independently of the presence of glucose (Table 1).

The maximum ectoine contents obtained were 14.9% and 15.1% in STR6_GLC and 22.9% and 21.7% in STR12_GLC (Fig.5). Hydroxyectoine was detected in both reactors at very low concentrations (maximum of 1.7% at 12% NaCl and 0.08% at 6% NaCl). Upon glucose depletion bacteria started to consume the intracellular ectoine and probably cell lysis occurred due to osmotic pressure. By day 10, intracellular ectoine concentrations stabilized in both conditions and were similar to those observed with CO₂ as sole carbon source. This was concomitant to a drop in the biomass concentration and CO₂-EC (Table 1).

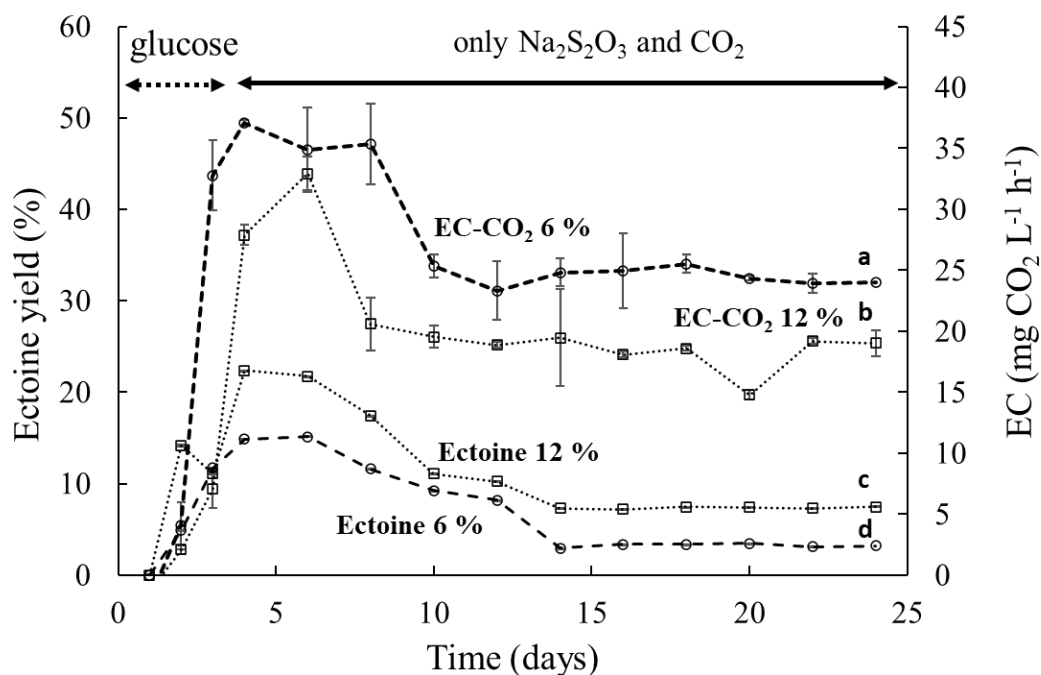


Fig.5. Time course of the CO₂ abatement and ectoine production during the operation with discontinuous glucose addition in Test 4. Lines with different letters were significantly different between them at $p < 0.05$.

When very small concentrations of glucose were added the bacteria grew in both CO₂ and glucose and the ectoine contents were comparable to those reported for *Halomonas* species growing in sugars, yeast and peptones as carbon sources (Liu et al., 2021).

Using glucose and CO₂ as carbon source ensures the assimilation of this GHG and the production of higher concentrations of ectoine and biomass, Sorokin et al. (2003, 2013) previously demonstrated that the addition of thiosulphate increases the endogenous ATP level during the oxidation of thiosulphate and the anaplerotic assimilation of CO₂ in *Halomonas* strains. In this regard, a biotechnological approach of mixotrophy where glucose is added in discontinuous and CO₂ in continuous could help to reduce costs in the industrial production of ectoines and can act as a CO₂ mitigation strategy. Moreover, the use of seawater as media, that contains organic compounds, high nitrate concentrations,

and high salinity should be also tested as a cheap resource to biotransform CO₂ into ectoines using *Halomonas*.

4. Conclusions

This study shows the feasibility of coupling ectoine production with the continuous abatement of CO₂. The biomass and ectoine contents obtained under thiosulfate degradation and CO₂ abatement (2.3 to 7.3 % g ectoine·g biomass⁻¹) were low compared to those obtained by *Halomonas* species using glucose and glutamate. However, the productivities of ectoine and CO₂ abatement can be improved through the discontinuous addition of organic compounds. In this study, the addition of low concentrations of glucose (0.5 g L⁻¹) increased ectoine contents to 15 and 22 % at salinities of 6 and 12 % NaCl and doubled the abatement of CO₂ in both conditions. Although, the organic compound tested in this research was glucose, the use of different organic compounds, such as acetate and alcohols that are usually treated as waste products in fermentations, could enhance this process by reducing costs. Overall, even though this biotechnology still requires improvement, this research opens the door to new CO₂ mitigation and bioproduction systems based on extremophile microorganisms that can enhance the development and implementation of circular strategies to abate carbon dioxide.

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6. Data Availability

Datasets related to this article can be found at NCBI (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA689702>) and at an open-source online data repository hosted at Mendeley Data.

7. References

- Ahmad, N., Ahmad, F., Khan, I., Khan, A.D., 2015. Studies on the Oxidative Removal of Sodium Thiosulfate from Aqueous Solution. *Arab. J. Sci. Eng.* 40, 289–293. <https://doi.org/10.1007/s13369-014-1473-0>
- Amulya, K., Venkata Mohan, S., 2022. Green hydrogen based succinic acid and biopolymer production in a biorefinery: Adding value to CO₂ from acidogenic fermentation. *Chemical Engineering Journal* 429, 132163. <https://doi.org/10.1016/j.cej.2021.132163>
- Anand, A., Raghuvanshi, S., Gupta, S., 2020. Trends in Carbon Dioxide (CO₂) Fixation by Microbial Cultivations. *Current Sustainable/Renewable Energy Reports* 7, 40–47. <https://doi.org/10.1007/s40518-020-00149-1>
- Becker, J., Wittmann, C., 2020. Microbial production of extremolytes — high-value active ingredients for nutrition, health care, and well-being. *Current Opinion in Biotechnology* 65, 118–128. <https://doi.org/10.1016/j.copbio.2020.02.010>
- Bose, A., Lin, R., Rajendran, K., O'Shea, R., Xia, A., Murphy, J.D., 2019. How to optimise photosynthetic biogas upgrading: a perspective on system design and microalgae selection. *Biotechnology Advances* 37, 107444. <https://doi.org/10.1016/j.biotechadv.2019.107444>
- Cantera, S., Fischer, P.Q., Sánchez-Andrea, I., Marín, D., Sousa, D.Z., Muñoz, R., 2021. Impact of the algal-bacterial community structure, physio-types and biological and environmental interactions on the performance of a high rate algal pond treating biogas and wastewater. *Fuel* 302, 121148. <https://doi.org/10.1016/j.fuel.2021.121148>
- Cantera, S., Lebrero, R., Rodríguez, E., García-Encina, P.A., Muñoz, R., 2017a. Continuous abatement of methane coupled with ectoine production by *Methylobacterium alcaliphilum* 20Z in stirred tank reactors: A step further towards greenhouse gas biorefineries. *Journal of Cleaner Production* 152, 134–141. <https://doi.org/10.1016/j.jclepro.2017.03.123>
- Cantera, S., Lebrero, R., Rodríguez, S., García-Encina, P.A., Muñoz, R., 2017b. Ectoine bio-milking in methanotrophs: A step further towards methane-based bio-

- refineries into high added-value products. *Chemical Engineering Journal* 328, 44–48. <https://doi.org/10.1016/j.cej.2017.07.027>
- Cantera, S., Lebrero, R., Sadornil, L., García-Encina, P.A., Muñoz, R., 2016. Valorization of CH₄ emissions into high-added-value products: Assessing the production of ectoine coupled with CH₄ abatement. *Journal of Environmental Management* 182, 160–165. <https://doi.org/10.1016/j.jenvman.2016.07.064>
- Cantera, S., Muñoz, R., Lebrero, R., López, J.C., Rodríguez, Y., García-Encina, P.A., 2018. Technologies for the bioconversion of methane into more valuable products. *Current opinion in biotechnology* 50, 128–135.
- Chen, Q., Zhang, L., Li, X., Liu, S., Li, D., 2014. Poly- β -hydroxybutyrate/ectoine co-production by ectoine-excreting strain *Halomonas salina*. *Process Biochemistry* 49, 33–37. <https://doi.org/10.1016/j.procbio.2013.09.026>
- Chen, W.-C., Hsu, C.-C., Wang, L.-F., Lan, J.C.-W., Chang, Y.-K., Wei, Y.-H., 2019. Exploring useful fermentation strategies for the production of hydroxyectoine with a halophilic strain, *Halomonas salina* BCRC 17875. *Journal of Bioscience and Bioengineering* 128, 332–336. <https://doi.org/10.1016/j.jbiosc.2019.02.015>
- Corti Monzón, G., Nisenbaum, M., Herrera Seitz, M.K., Murialdo, S.E., 2018. New Findings on Aromatic Compounds' Degradation and Their Metabolic Pathways, the Biosurfactant Production and Motility of the Halophilic Bacterium *Halomonas* sp. KHS3. *Current Microbiology* 75, 1108–1118. <https://doi.org/10.1007/s00284-018-1497-x>
- Czech, L., Hermann, L., Stöveken, N., Richter, A.A., Höppner, A., Smits, S.H.J., Heider, J., Bremer, E., 2018. Role of the Extremolytes Ectoine and Hydroxyectoine as Stress Protectants and Nutrients: Genetics, Phylogenomics, Biochemistry, and Structural Analysis. *Genes* 9, 177. <https://doi.org/10.3390/genes9040177>
- Dia, M., Wehner, T.C. and Arellano, C. (2017) RGxE: An R Program for Genotype x Environment Interaction Analysis. *American Journal of Plant Sciences*, **8**, 1672–1698. doi: [10.4236/ajps.2017.87116](https://doi.org/10.4236/ajps.2017.87116).
- Gabrielli, P., Gazzani, M., Mazzotti, M., 2020. The Role of Carbon Capture and Utilization, Carbon Capture and Storage, and Biomass to Enable a Net-Zero-CO₂ Emissions Chemical Industry. *Ind. Eng. Chem. Res.* 59, 7033–7045. <https://doi.org/10.1021/acs.iecr.9b06579>
- García-González, L., Mozumder, Md.S.I., Dubreuil, M., Volcke, E.I.P., De Wever, H., 2015. Sustainable autotrophic production of polyhydroxybutyrate (PHB) from CO₂ using a two-stage cultivation system. *Catalysis Today* 257, 237–245. <https://doi.org/10.1016/j.cattod.2014.05.025>
- Hobmeier, K., Goëss, M.C., Sehr, C., Schwaminger, S., Berensmeier, S., Kremling, A., Kunte, H.J., Pflüger-Grau, K., Marin-Sanguino, A., 2020. Anaplerotic Pathways in *Halomonas elongata*: The Role of the Sodium Gradient. *Frontiers in Microbiology* 11, 2124. <https://doi.org/10.3389/fmicb.2020.561800>
- IPCC, 2018: Summary for Policymakers. In: *Global Warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty* [Masson-Delmotte, V., P. Zhai, H.-O. Pörtner, D. Roberts, J. Skea, P.R. Shukla, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J.B.R. Matthews, Y. Chen, X. Zhou, M.I.

Gomis, E. Lonnoy, T. Maycock, M. Tignor, and T. Waterfield (eds.)). World Meteorological Organization, Geneva, Switzerland, 32 pp

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 *Methylobacterium* and *Methylobacter* and emended description of the genus *Methylobacterium*. *International Journal of Systematic and Evolutionary Microbiology* 58, 591–596.

Khandelwal, A., Anand, A., Raghuvanshi, S., Gupta, S., 2021. Integrated approach for microbial carbon dioxide (CO₂) fixation process and wastewater treatment for the production of hydrocarbons: Experimental studies. *Journal of Environmental Chemical Engineering* 9, 105116. <https://doi.org/10.1016/j.jece.2021.105116>

Kumar, M., Sundaram, S., Gnansounou, E., Larroche, C., Thakur, I.S., 2018. Carbon dioxide capture, storage and production of biofuel and biomaterials by bacteria: A review. *Bioresource Technology* 247, 1059–1068. <https://doi.org/10.1016/j.biortech.2017.09.050>

Kumar, Manish, Kumar, Madan, Pandey, A., Thakur, I.S., 2019. Genomic analysis of carbon dioxide sequestering bacterium for exopolysaccharides production. *Scientific Reports* 9, 4270. <https://doi.org/10.1038/s41598-019-41052-0>

Kumar, V., Thakur, I.S., 2020. Biodiesel production from transesterification of *Serratia* sp. ISTD04 lipids using immobilised lipase on biocomposite materials of biomineralized products of carbon dioxide sequestering bacterium. *Bioresource Technology* 307, 123193. <https://doi.org/10.1016/j.biortech.2020.123193>

Kunte, H., Lentzen, G., Galinski, E., 2014. Industrial Production of the Cell Protectant Ectoine: Protection Mechanisms, Processes, and Products 3, 10–25. <https://doi.org/10.2174/22115501113026660037>

Liu, M., Liu, H., Shi, M., Jiang, M., Li, L., Zheng, Y., 2021. Microbial production of ectoine and hydroxyectoine as high-value chemicals. *Microbial Cell Factories* 20, 76. <https://doi.org/10.1186/s12934-021-01567-6>

Metz, B., Davidson, O., de Coninck, H., Loos, M., Meyer, L., 2018. Sources of carbon dioxide, in: *Carbon Dioxide Capture and Storage*, IPCC. Cambridge University Press, p. 431.

Mishra, S., Pahari, S., K, S., Mohanty, S., Gupta, S., Raghuvanshi, S., 2018. Investigation on CO₂ bio-mitigation using *Halomonas stevensii* in laboratory scale bioreactor: Design of downstream process and its economic feasibility analysis. *Journal of CO₂ Utilization* 24, 274–286. <https://doi.org/10.1016/j.jcou.2018.01.018>

Mishra, S., Raghuvanshi, S., Gupta, S., Raj, K., 2017. Application of novel thermo-tolerant haloalkalophilic bacterium *Halomonas stevensii* for bio mitigation of gaseous phase CO₂: Energy assessment and product evaluation studies. *Process Biochemistry* 55, 133–145. <https://doi.org/10.1016/j.procbio.2017.01.019>

Molitor, B., Mishra, A., Angenent, L.T., 2019. Power-to-protein: converting renewable electric power and carbon dioxide into single cell protein with a two-stage bioprocess. *Energy Environ. Sci.* 12, 3515–3521. <https://doi.org/10.1039/C9EE02381J>

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. *Biotechnology Advances* 28, 782–801. <https://doi.org/10.1016/j.biotechadv.2010.06.005>
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research* 41, D590–D596. <https://doi.org/10.1093/nar/gks1219>
- Sauer, T., Galinski, E.A., 1998. Bacterial milking: A novel bioprocess for production of compatible solutes. *Biotechnology and Bioengineering* 57, 306–313. [https://doi.org/10.1002/\(SICI\)1097-0290\(19980205\)57:3](https://doi.org/10.1002/(SICI)1097-0290(19980205)57:3)
- Schloss, P.D., 2020. Reintroducing mothur: 10 Years Later. *Appl. Environ. Microbiol.* 86. <https://doi.org/10.1128/AEM.02343-19>
- Smith, R.T., Bangert, K., Wilkinson, S.J., Gilmour, D.J., 2015. Synergistic carbon metabolism in a fast growing mixotrophic freshwater microalgal species *Micractinium inermum*. *Implement. Sustain. Bioenergy Syst. Insights* 2014 RCUK Int. Bioenergy Conf. 82, 73–86. <https://doi.org/10.1016/j.biombioe.2015.04.023>
- Song, C., Pan, W., Srimat, S.T., Zheng, J., Li, Y., Wang, Y.-H., Xu, B.-Q., Zhu, Q.-M., 2004. Tri-reforming of Methane over Ni Catalysts for CO₂ Conversion to Syngas With Desired H₂/CO Ratios Using Flue Gas of Power Plants Without CO₂ Separation, in: Park, S.-E., Chang, J.-S., Lee, K.-W. (Eds.), *Studies in Surface Science and Catalysis*. Elsevier, pp. 315–322. [https://doi.org/10.1016/S0167-2991\(04\)80270-2](https://doi.org/10.1016/S0167-2991(04)80270-2)
- Sorokin, D.Yu., 2003. Oxidation of Inorganic Sulfur Compounds by Obligately Organotrophic Bacteria. *Microbiology* 72, 641–653. <https://doi.org/10.1023/B:MICL.0000008363.24128.e5>
- Sorokin, D.Y., Banciu, H., Robertson, L.A., Kuenen, J.G., Muntyan, M.S., Muyzer, G., 2013. Halophilic and Haloalkaliphilic Sulfur-Oxidizing Bacteria, in: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), *The Prokaryotes: Prokaryotic Physiology and Biochemistry*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 529–554. https://doi.org/10.1007/978-3-642-30141-4_7
- Vavourakis, C.D., Mehrshad, M., Balkema, C., van Hall, R., Andrei, A.-Ş., Ghai, R., Sorokin, D.Y., Muyzer, G., 2019. Metagenomes and metatranscriptomes shed new light on the microbial-mediated sulfur cycle in a Siberian soda lake. *BMC Biology* 17, 69. <https://doi.org/10.1186/s12915-019-0688-7>
- Yang, X., Xu, M., Zou, R., Angelidaki, I., Zhang, Y., 2021. Microbial protein production from CO₂, H₂, and recycled nitrogen: Focusing on ammonia toxicity and nitrogen sources. *Journal of Cleaner Production* 291, 125921. <https://doi.org/10.1016/j.jclepro.2021.125921>
- Zeng, A.-P., 2019. New bioproduction systems for chemicals and fuels: Needs and new development. *Biotechnology Advances* 37, 508–518. <https://doi.org/10.1016/j.biotechadv.2019.01.003>
- Zhang, L., Lang, Y., Nagata, S., 2009. Efficient production of ectoine using ectoine-excreting strain. *Extremophiles* 13, 717. <https://doi.org/10.1007/s00792-009-0262-2>