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Enhanced ectoines 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 CO_2 as the added carbon source, and CO_2 and glucose. Initially, CO_2 alone was fed to continuous reactors, adding thiosulphate as the energy source. Maximum CO_2 elimination capacities of 24.2 mg CO_2 L^{-1} h^{-1} were obtained, and ectoine contents up to 7.3% (g ectoine·g biomass^{-1}). To enhance ectoine production, CO_2 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 CO_2 removal to 37.1 mg CO_2 L^{-1} h^{-1} and increased ectoine contents up to 22%. Our results represent the proof of concept for a CO_2 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 CO_2.

1. Introduction

Carbon dioxide (CO_2) emissions represent approximately 82% of the total greenhouse gases (GHGs) released worldwide, and their share is expected to increase in the coming years [19,27]. To overcome this problem, current research and political initiatives focus on building a circular, resource-efficient and climate-resilient society [3]. Recycling of CO_2 for the production of industrially valuable products has become one of the big pillars to achieve this circularity [16].

Energy and industrial sectors are the main sources of anthropogenic CO_2 emissions with an annual dischraged of 666 Mt of CO_2 to the atmosphere. CO_2 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% CO_2) or coal-fired boilers (12–14% CO_2) [36]. This CO_2 can be used as feedstock for the production of materials, chemicals and fuels. In principle, the physico-chemical transformation of CO_2 is unattractive because of i) the high requirements of energy and chemicals, and ii) the technical and regulatory limitations for the logistics of CO_2 since these technologies usually require prior CO_2 capture, concentration, removal of impurities, and transportation to the application sites [22]. A viable, low energy-demanding option to transform CO_2 directly from flue gases, without prior accumulation or pre-treatment, entails the use of the catalytic activity of specialized microorganisms [16,21]. CO_2 can be assimilated as cellular biomass and transformed into products with market value. So far, the use of CO_2 as a substrate to produce valuable chemicals relays mainly on the use of algae, which requires large implementation areas for photobioreactors [5]. In the case of dark CO_2 fixation, promising cell factories are being researched to produce bioplastics, biodiesels, single cell protein and extracellular polysaccharides [2,17,23,24,30,40]. 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 [41]. Thus, the expansion of both, the portfolio of valuable products synthesized from CO_2 and, the diversity of organisms used for CO_2 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} [4]. 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 [26], which makes of them a target product in the pharmaceutical industry [4]. 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,

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inflating production cost [10,25].

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 [11,12,42]. Recent studies have shown that *Halomonas stevensii* can grow using thiosulfate as the energy donor without an extra apparent carbon source rather than CO_2 [21,28,29], however, to date, CO_2 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 [1].

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 CO_2 incorporation and thiosulfate removal. CO_2 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 CO_2 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}): KNO_3 - 1, K_2HPO_4 - 1, $MgSO_4 \bullet 7H_2O$ - 0.2, $CaCl_2 \bullet 2H_2O$ - 0.02, NH_4Cl_2 – 0.16 and trace elements (mg L^{-1}): ($CuCl_2 \bullet 5\ H_2O$ - 0.1, $FeSO_4 \bullet 7H_2O$ - 2, ZnSO4×7 H2O - 0.1, $NiCl_2 \bullet 6H_2O$ - 0.02, $CoCl_2$ x $6H_2O$ - 0.2, Na_2MoO_4 - 0.3, $MnCl_2$ x $4H_2O$ - 0.03, H_3BO_3 - 0.03). MSM was autoclaved at 1.5 atm at 121°C for 20 min. $Na_2S_2O_3$ 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 $Na_2S_2O_3$. Bottles were closed using gas-tight butyl septa and aluminum caps. CO_2 was then injected to the headspace at an initial concentration of 20/80 (v/v) CO_2/air (CO_2 was added after removal of 20% of air to maintain atmospheric pressure). The cultures were incubated at 30 or 37 ºC (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) CO_2/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. [9]. 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 ºC using a Spherisorb Amino (NH_2) Column, 80 Å, 3 μm, 4.6 mm × 150 mm (Waters, USA). The mobile phase consisted in acetonitrile/H_2O 75/25 (%) at a flow rate of 1 mL min^{-1}. Ectoine and hydroxyectoine 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 [(4 S,5 S)− 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 ℃ 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 C_{18} 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 H_2SO_4 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).

CO_2 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 ºC, respectively. Helium was used as carrier gas at 13.7 mL min^{-1}. The CO_2 elimination capacity (CO_2-EC) was calculated using Eq. (1).

$$CO2 - EC = \frac{Q \times ([CO2]in - [CO2]out)}{VR} \quad (1)$$

where Q= inlet gas flow, [CO_2]in= CO_2 inlet concentration, [CO_2]out= CO_2 outlet concentration, VR= Reactor volume.

Optical absorbance measurements at 600 nm (OD_{600}) 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. CO_2 uptake by Halomonas stevensii - test 1

Test 1 was performed to assess the growth of *H. stevensii* in batch bottles where CO_2 was the only carbon source provided. In Test 1, 1 mL of *H. stevensii* culture acclimatized to CO_2 (Section 2.2) was transferred to triplicate 120 mL serum bottles filled with 50 mL of MSM supplemented with 10 mM $Na_2S_2O_3$. Prior inoculation, 12 mL of air were removed from the bottles and the same amount of CO_2 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 CO_2 abatement.

2.5. Ectoine production enhanced by CO_2 assimilation – test 2

Test 2 was carried out as first proof of concept of ectoine production associated with CO_2 elimination in continuous bioreactors. For this, four 1-L Eppendorf stirred-tank reactors (STR_S) 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 $Na_2S_2O_3$. A 0.016 ± 0.005 L min^{-1} CO_2-laden air stream containing 360.8 ± 18 mg CO_2 L (≈ 20%; CO_2 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 CO_2 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 min. 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 CO_2 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 OD_{600}, 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 OD_{600} and TSS, sulphur species, ectoine, and hydroxyectoine.

2.6. Influence of salinity in ectoine contents and CO_2 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 ± 0.001 L min^{-1} CO_2-laden air stream containing 360.5 ± 20.2 mg CO_2 L^{-1} (≈ 20%), corresponding to a CO_2 load of 373.2 ± 25.7 mg CO_2 L^{-1} 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 CO_2 elimination capacity (CO_2-EC) deviated < 10% from the mean. 5 mL of culture broth were removed from the bioreactors on days 1, 3 and 5 to determine OD_{600} 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 OD_{600}, TSS, DIC, organic acids, sulphate, sulphite, sulphur and thiosulphate, ectoine and hydroxyectoine. Gas samples for CO_2 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).

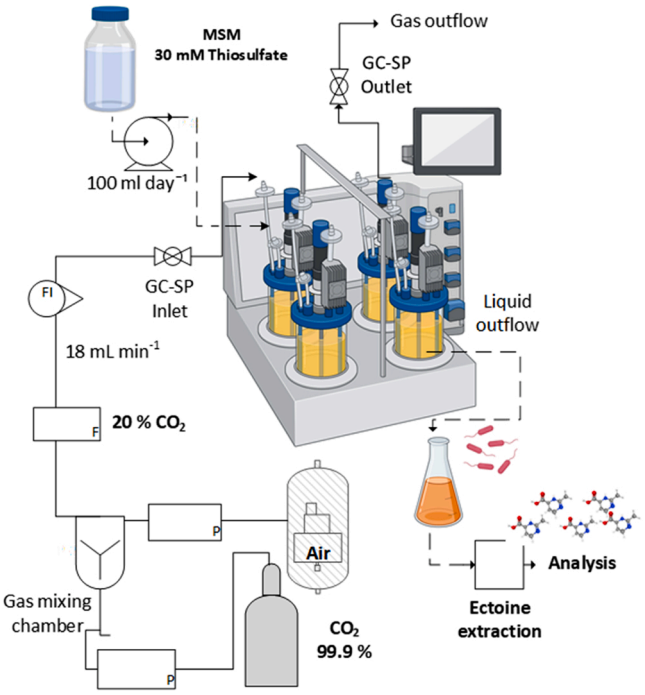

Fig. 1. Schematic diagram of the experimental setup: Pressure meter (P), Flow mass controller (F), Rotameter (FI), Mineral salt media (MSM) storage tank, Gas streams (continuous line), Liquid streams (discontinuous line), Gas chromatograph sampling port (GC-SP).

2.7. Influence of glucose addition on CO_2 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 CO_2 abatement by *H. stevensii*. The selection of the glucose concentration was based on the external carbon requirements for other mixotrophic bacteria [35]. 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 OD_{600}, 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 OD_{600}, TSS, IC, sulphate, sulphite and thiosulphate, as well as ectoine and hydroxyectoine. Gas samples for CO_2 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 16 S rRNA gene (Sanger sequencing). 16 S 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 ºC for 10 min; 35 cycles at 95 ºC for 30 s, 55 ºC for 40 s, and 72 ºC for 90 s; and a final elongation cycle at 72 ºC 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 16 S 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, 16 S 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 16 S rRNA gene, was used [43]. The 16 S rRNA gene sequences were processed and quality filtered using Mothur v1.44.3 following the Mother SOP (https://www.mothur.org/wiki/MiSeq_SOP) [34]. Sequences were then clustered at 97% identity threshold into Operational Taxonomic Units (OTUs) using the SILVA 16 S rRNA gene reference database (Version: 138.1) [32]. Microbial diversity was analyzed using R version 1.4.1 [15] and heatmaps plotted using the package *pheatmap* (R package version 1.0.12) (Supplementary Figs. S1 and S2). All sequences obtained (deposited at GenBank as bio-project: 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. [6]. Analytical results are given as the average of biological replicas ± standard deviation and technical replicas ± standard deviation depending on the experiment.

3. Results and discussion

3.1. CO_2 elimination by Halomonas stevensii

Test 1 consisted on batch cultivations to test if members of the ectoine producing *Halomonas* genus were able to eliminate CO_2 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 CO_2 (79.8 ± 7.7 mg CO_2 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 hypothethise 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 CO_2 depletion, thus the removal of CO_2 from the headspace in *H. stevensii* cultures was linked to biological activity.

The pH value in the incubations increased to 7.9 due to CO_2 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 CO_2 elimination in the headspace. At pH 8.3 and 9, CO_2 was sequestrated in the liquid phase as carbonate and could not be detected in the headspace.

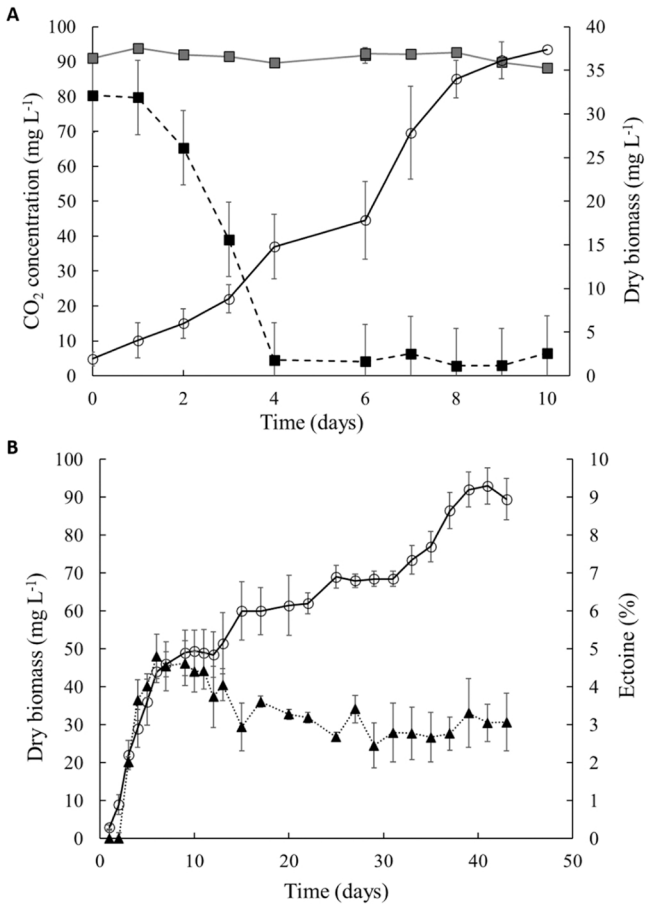

Fig. 2. A) Growth and CO_2 abatement by H. stevensii in batch bottles, Test 1, using 10 mM of $Na_2S_2O_3$ as energy source and pH of 7 (data shown as mean of triplicates (±) standard deviation (mean ± SD)) B) Growth of H. stevensii and ectoine production in four independent continuous bioreactors in Test 2. 20 mM of $Na_2S_2O_3$ were used as energy source (data shown as mean of quadruplicates (±) standard deviation (mean ± SD)). CO_2 was the only carbon source supplied in both batch bottles and bioreactor experiments. Grey line: abiotic control, ○-black line: biomass concentration, ■-dashed line: CO_2 concentration, ▲-dotted line: ectoine accumulation.

Previously, Mishra et al. [29] had already observed complete CO_2 depletion (of 10% v/v CO_2 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}) [28,29]. These high biomass yields could be due to the higher available CO_2 in the liquid broth at the higher pH used, or to the presence of an unknown carbon source in the cultures that could be growing in mixotrophy.

3.2. Ectoine production from thiosulphate and CO_2

Sterile bioreactors continuously fed with CO_2 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 OD_{600} and pH did not vary (0.005 ± 0.003 and 7.45 ± 0.05, respectively) and thiosulfate and sulphate concentrations were constant (15.1 ± 0.6 mM $S_2O_3^{2-}$ and 0.8 ± 0.1 mM SO_4^{2-}). In the biotic reactors, the biomass concentration 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 $S_2O_3^{2-}$ detected in the media throughout

operation) with the production of sulphate (12.3 ± 0.5 mM SO_4^{2-}) and sulphur ~3–6 mM S^0 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 ± 0.6% on dry weight basis). Intracellular 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 [14]. During steady state, ectoine contents obtained were 3.3 ± 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% [4]. In addition to glucose being a better carbon source, most of previous studies used a high-salinity medium (NaCl concentrations from 15% to 20%), which favours ectoine production [4,31].

3.3. Influence of salinity on CO_2 bioconversion

Higher salinities (6–20% NaCl) and sodium thiosulphate concentrations (30 mM) were used in a follow up experiment, Test 3. No significant CO_2 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 CO_2 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 CO_2 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-CO_2 obtained in STR_6 (23.9 ± 2.9 mg L^{-1} h^{-1}) was statistically equal to the one recorded in STR_9 (22.4 ± 2.7 mg L^{-1} h^{-1}). At 12% NaCl, the bacterial performance in CO_2 removal was significantly reduced (17.5 ± 1.9 mg L^{-1} h^{-1}) and at 20% NaCl, the high salinity concentration probably hindered bacterial growth and the EC-CO_2 was negligible (Table 1, Fig. 3).

These results were aligned with the biomass concentration achieved (Table 1, Fig. 3). The biomass obtained was statistically higher in STR_6 and STR_9 (236.5 ± 16.4 and 239.2 ± 37.4 mg L^{-1}, respectively). In fact, the biomass obtained at 6% NaCl was higher than the one found in the previous operation (92.5 ± 4.8 mg L^{-1}). We correlated this result with the higher amounts of S_2O_3 available for the bacteria due to the higher dilution rates and with the potential removal of toxic sulphur species. In STR_12, the biomass concentration under steady state was significantly lower (179.6 ± 11.5 mg L^{-1}). Although dry biomass values were very low (26.8 ± 6.3 mg biomass L^{-1}) in STR_20, high OD_{600} were recorded. Microscope observations corroborated that there were almost no floating cells but orange precipitates that were accumulating in this bioreactor, probably caused by the high salt concentrations. According to the carbon balance analyses, the biomass hourly produced in steady state (Table 1) showed that around 10% of the carbon from CO_2 went to biomass in STR_6, STR_9 and STR_12, around 0.5% went to the production of organic acids (supplementary materials, Table S2) that were excreted to the medium, and the rest remained as bicarbonate/carbonate in the liquid phase.

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

Mishra et al. [28] operated reactors in continuous with *H. stevensii* (15% of CO_2 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 CO_2 added, however sulphur species were not measured in their study [28]. Members of the *Halomonas* genus have been identified as able to oxidize thiosulphate in similar rates than sulphur oxidizing bacteria [39]. However, this oxidation of thiosulphate was partial and the product obtained was tetrathionate that in neutral conditions decomposes to sulphate and sulphur [37]. 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. [28,29]. 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 CO_2 assimilation [38], 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) [37]. In this regard, it seems plausible that *Halomonas* species require mixotrophy to efficiently assimilate CO_2 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 concentration 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 [7,11], 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 [9,20]. 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%).

As expected higher ectoines 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 CH_4 as the sole carbon source (7–11%) [8,9]. 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% and 20% of intracellular ectoine and 2.0–3.5% of hydroxyectoine at salinities of 15–20% NaCl using glucose or glutamate as the main substrate [4,26,31,33]. These results showed that the bacteria were able to survive and maintain minimum cell metabolism with thiosulphate and CO_2, 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 CO_2 assimilation is increased in the presence of organic substrates [37]. Therefore, a good strategy to increase ectoine production and CO_2 elimination while diminishing the

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})	$S_2O_3^{-2}$(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.

costs of this biotechnology could be the discontinuous addition of low concentrations of more energetic organic sources, such as glucose, acetate or alcohols [13,18].

3.5. Influence of glucose addition on ectoine production and CO_2 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 h, 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 CO_2 and an EC-CO_2 of 32.9 ± 1.9 mg CO_2 L^{-1} h^{-1} was recorded (202.8 mg biomass L^{-1} day would be the potential biomass from the total CO_2 consumed considering that 50% of the carbon is diverted to metabolism). After 24 h (day 4) the biomass achieved values of 396.1 mg biomass L^{-1} and an EC-CO_2 37.1 ± 2.9 mg CO_2 L^{-1} h^{-1} probably using the energy of thiosulphate and polyhydroxyalkanoate and ectoine catabolism. Once glucose was completely depleted (STR6_CO_2) the biomass and the EC-CO_2 dropped and stabilized in values similar to those obtained in STR_6 with CO_2 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 h 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 [25,31]. An EC-CO_2 of 27.8 ± 0.8 mg CO_2 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-CO_2 of 32.9 ± 1.4 mg CO_2 L^{-1} h^{-1} mg. In STR12_CO_2, the biomass and the EC-CO_2 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 CO_2 as sole carbon source. This was concomitant to a drop in the biomass concentration and CO_2-EC (Table 1).

When very small concentrations of glucose where added the bacteria grew in both CO_2 and glucose and the ectoine contents were comparable to those reported for *Halomonas* species growing in sugars, yeast and peptones as carbon sources [26].

Using glucose and CO_2 as carbon source ensures the assimilation of this GHG and the production of higher concentrations of ectoine and biomass, Sorokin et al. [38] previously demonstrated that the addition of thiosulphate increases the endogenous ATP level during the oxidation of thiosulphate and the anaplerotic assimilation of CO_2 in *Halomonas* strains. In this regard, a biotechnological approach of mixotrophy where glucose is added in discontinuous and CO_2 in continuous could help to reduce costs in the industrial production of ectoines and can act as a CO_2 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 CO_2 into ectoines using *Halomonas*.

4. Conclusions

This study shows the feasibility of coupling ectoine production with the continuous abatement of CO_2. The biomass and ectoine contents obtained under thiosulfate degradation and CO_2 abatement (2.3–7.3% g

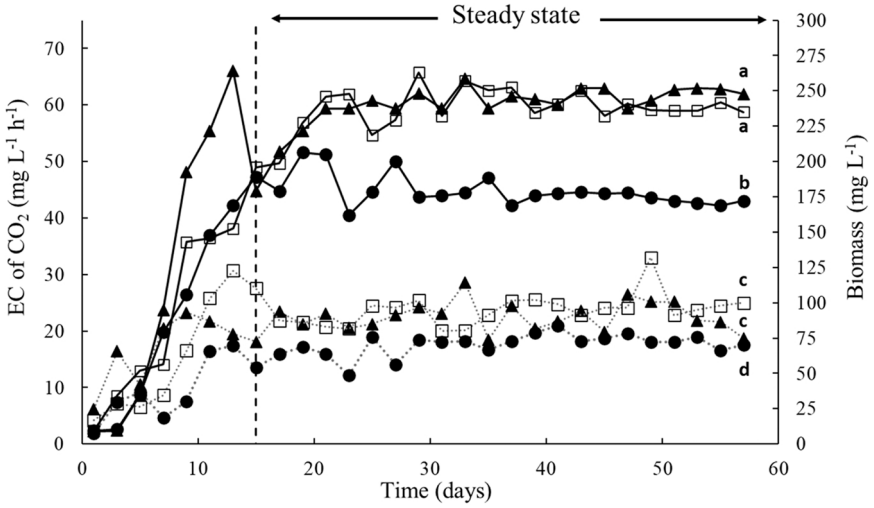

Fig. 3. Represents the results of Test 3. Time course of the EC-CO_2 at: 6% NaCl (□-dotted line), 9% NaCl, (▲-dotted line) and 12% NaCl (●-dotted line). Time course of the biomass concentration at: 6% NaCl (□-black line), 9% NaCl(▲-black line) and 12% NaCl (●-black line). Different letters at the end of each line show significant difference between average values of each condition at $p < 0.05$.

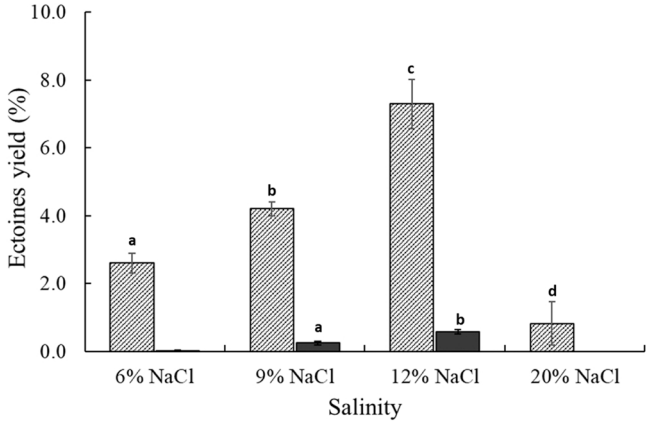

Fig. 4. Influence of the concentration of NaCl on the steady state contents obtained of intra-cellular ectoine (dashed column) and hydroxyectoine (grey column) in Test 3. Vertical lines represent standard deviations from replicates. Columns intra-groups with different letters were significantly different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

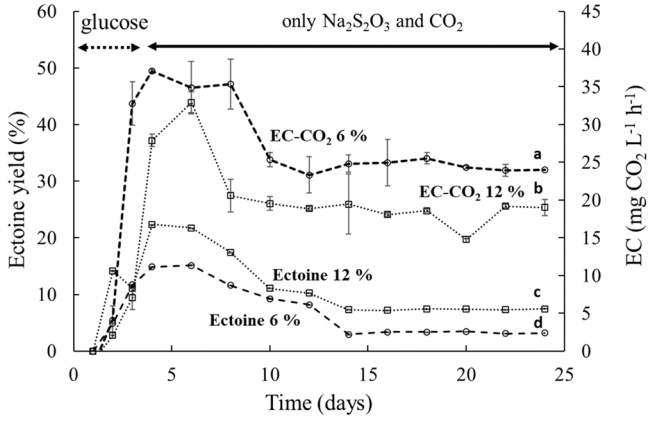

Fig. 5. Time course of the CO_2 abatement and ectoine production during the operation with discontinuous glucose addition in Test 4. Lines with different letters were significantly different between them at $p < 0.05$.

ectoine·g biomass^{-1}) were low compared to those obtained by *Halomonas* species using glucose and glutamate. However, the productivities of ectoine and CO_2 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 CO_2 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 CO_2 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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CRediT authorship Contribution Statement

Dr. Cantera was responsible for the conception and design of the research and article and interpretation of the data and drafting of the manuscript. Dr. Sousa and Dr. Sanchez-Andrea developed the critical revision of the article to provide with important intellectual content. All authors read and approved this manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jcou.2022.102009.

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