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Guyparkeria halophila: Novel cell platform for the efficient valorization of carbon dioxide and thiosulfate into ectoine

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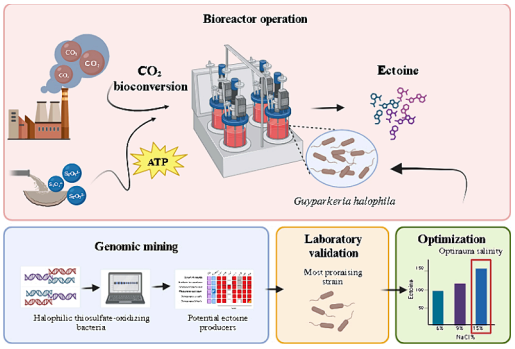
HIGHLIGHTS

- Proof of a novel CO_2 and $S_2O_3^{-2}$ valorization platform for ectoines.
- Genome mining allowed to find 38 potential strains for CO_2 and $S_2O_3^{-2}$ valorization.
- Four strains were validated in the laboratory to produce ectoines.
- Process was optimized with *G. halophila* in batch and continuous bioreactors.
- 47 % of specific ectoine content was achieved, among the highest in natural organisms.

GRAPHICAL ABSTRACT



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ABSTRACT

Utilizing carbon dioxide (CO_2) for valuable chemical production is key to a circular economy. Current processes are costly due to limited microorganism use, low-value products, and the need for affordable energy. This study addresses these challenges by using industrial contaminants like thiosulfate ($S_2O_3^{2-}$) for CO_2 conversion into ectoines. Ectoines, are important ingredients as pharmaceuticals and cosmetics. Here, six microbial genomes were identified as potential candidates to valorize CO_2 and $S_2O_3^{2-}$ into ectoine. After laboratory validation at 3 % NaCl, the fastest-growing strain, Guyparkeria halophila, was optimized at 6 %, 9 %, and 15 % NaCl, showing the highest specific ectoine contents ($mg_{Ect}\ g^{-1}_{biomass}$) at 15 %. Batch bioreactors, combining optimal conditions, achieved maximum specific ectoine contents of 47 %. These results not only constitute the highest ectoine content so far reported by autotrophs and most of heterotrophs, but also the first proof of a novel valorization platform for CO_2 and $S_2O_3^{2-}$, focused on pharmaceuticals production.

1. Introduction

Mitigating carbon dioxide (CO_2) emissions holds crucial significance in tackling the pressing challenges presented by climate change. CO_2 stands out as the foremost anthropogenic greenhouse gas (71.6 %, data from 2021, EEA European Environmental Agency), being the principal

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contributor in fostering global warming and ensuing detrimental environmental outcomes (Villora-Picó et al., 2024). In 2022, global CO_2 emissions rose by 1.5 % compared to 2021, resulting in a concerning amount of 37.15 Gt of CO_2 (Min et al., 2022). According to the International Energy Agency (IEA), CO_2 emissions from energy combustion and industrial processes accounted for 89 % of greenhouse gas emissions in 2022, highlighting them as the primary contributors of anthropogenic worldwide CO_2 emissions (Al-Ismail et al., 2023).

In this scenario, the convergence of scientific and industrial efforts has been directed towards advancing novel bio-production systems that rely on renewable resources, with a specific focus on mitigating greenhouse gases (Bachleitner et al., 2023). A pivotal strategy in achieving this sustainability and fostering cleaner production systems involves the valorization of CO_2 emissions (Onyeaka and Ekwebelem, 2023). Remarkably, CO_2 serves as a non-toxic, non-flammable and cost-effective carbon source, acting as a fundamental building block for synthesizing value chain chemicals. Its abundance is virtually limitless, constituting a nearly cost-free carbon source derived from flue gases (Grim et al., 2022). A good approach to valorize CO_2 emissions from flue gasses is dark gas fermentation based on the use of chemolithoautotrophic organisms (Santos Correa et al., 2023). These biotechnologies are tailored to effective gas circularity and low intensity land-use and allow to produce a broad range of valuable compounds, including chemicals with high social and economic value (Gulati et al., 2023). Moreover, the energy necessary to promote CO_2 fixation can come directly from the source emission, since several polluting sectors also produce gas and liquid waste streams composed of contaminant co-products, such as carbon monoxide (CO), reduced sulfur species, ammonia, and metals that need to be treated prior to being discharged into the surrounding environment (Wu et al., 2024). In this context, dark fermentation emerges as a versatile approach, addressing both the reduction of CO_2 emissions and the concurrent removal of reduced industrial contaminants. Notably, the generated biomass becomes a resource for the production of valuable chemicals. Interesting bacteria able to fixate CO_2 and eliminate reduced contaminants are chemolithotrophic sulfur-oxidizing bacteria (SOB) known for the ability to oxidize reduced sulfur compounds as energy sources. These bacteria have already found applications in several environmental remediation strategies (Nguyen et al., 2022).

Thiosulfate ($S_2O_3^{-2}$), as a reduced sulfur compound, poses potential threats to both human well-being and ecosystems (Ryon et al., 2002). Wastewater discharges, particularly from industries such as mining, petroleum refining, leather and paper production, and coal processing, commonly contain elevated concentrations of $S_2O_3^{-2}$, exceeding 3000 mg L^{-1} (Ahmad et al., 2015). The untreated release of such discharges can lead to significant issues, including the corrosion of sewer pipes, eutrophication and the formation of hydrogen sulfide, among others (Pirieh and Naeimpoor, 2020). Currently, $S_2O_3^{-2}$ is widely employed as a source of energy in autotrophic denitrification processes (Fan et al., 2021) or soil remediation strategies (Bao et al., 2016). However, simultaneous transformation of CO_2 and $S_2O_3^{-2}$ could lead to unexplored opportunities, like producing valuable compounds such as pharmaceuticals and cosmetics. This shift towards alternative synthesis methods not only cuts costs but also encourages circular economy practices and innovative bio-production systems (Fan et al., 2021).

Ectoine is an osmolyte produced by halophiles under high osmotic stress environment in order to maintain osmotic equilibrium (Ng et al., 2023). Owing to its exceptional properties on humans (osmotic protection, radiation protection, moisturizing effects, enhancement of the immune defense capacity of skin cells and cellular repair) it is commonly used as key ingredient for pharmaceuticals and cosmetics (Xu et al., 2023). Currently, ectoine is one of the most lucrative products obtained from microorganisms (600–1000 € kg^{-1}). Therefore, it is already being manufactured in the bio-industry using sugars and sold to important pharmaceutical companies, including Johnson & Johnson, Merk and PARI GmbH (Becker and Wittmann, 2020). However, the potential for generating ectoine from renewable or waste-based carbon and energy sources has remained largely unexplored (Cantera et al., 2023).

The primary goal of this study is to target a completely new strategy that can pave the way for a more cost-effective, circular and sustainable production of ectoine from CO_2 using industrial contaminant byproducts such as thiosulfate. In this research CO_2 together with S_2O3^{-2} are valorized into ectoines by using unexplored halophilic thiosulfate-oxidizing bacteria. Several thiosulfate-oxidizing bacteria that contained the genes for ectoine synthesis were selected based on their genome capabilities. Laboratory validation of ectoine production was developed with the selected strains using CO_2 and $S_2O_3^{-2}$. The most promising bacteria, according to their growth rates and ectoine production were optimized under different salinities, and finally implemented in batch bioreactors to achieved high ectoine contents from CO_2 and $S_2O_3^{-2}$ removal.

2. Materials and methods

2.1. Selection of chemolithoautotrophic thiosulfate-oxidizing bacteria able to produce ectoine

The screening of potential halophilic and halotolerant chemolithotrophic thiosulfate-oxidizing bacteria was performed according to Cantera et al, 2022. Genome mining was performed on genomes of these identified strains gathered from the National Center for Biotechnology Information (NCBI, Datasets v. 10.0.0) and screened for the presence of genes responsible for the ectoine synthesis pathways: *ect*A, *ect*B, *ect*C, and *ect*D. Additionally, the presence of *ect*R, which encodes a MarR-type repressor protein (EctR) that is involved in the transcriptional control of a considerable number of ectoine biosynthetic gene clusters, along with two genes related to the generation of the precursor L-2,4-diaminobutyrate (namely, aspartate kinase: *ask* and aspartate semialdehyde dehydrogenase: *asd*), was evaluated (Czech et al., 2018). The identified thiosulfate-oxidizing strains: *Guyparkeria halophila* DSM 6132, *Salinisphaera hydrothermalis* DSM 21493, *Thiohalomonas denitrificans* DSM 15841, *Thiomicrospira aerophila* DSM 13739 were acquired from the DSMZ culture collection (Leibniz-Institut, Germany). All thiosulfate-oxidizing strains were purchased as freeze-dried cultures and activated following the culture media conditions described by DSMZ. Growth characteristics of each strain are found in Supplementary materials 1.

2.2. Chemicals and mineral salt medium

The ammonium mineral salt medium (ASM) used for the growth of the thiosulfate-oxidizing strains was composed of (g L^{-1}): $MgSO_4{\cdot}7H_2O$-1.0, $CaCl_2{\cdot}2H_2O$-0.11, NH_4Cl_2-0.5, KNO_3-1.0. Medium was supplemented with trace elements (mg L^{-1}): $CuCl_2$-0.01, $FeCl_2$-0.9, $ZnCl_2$-0.06, $NiCl_2$-0.01, $CoCl_2$-0.06, Na_2MoO_4-0.03, $MnCl_2$-0.06, H_3BO_3-0.06, Na_2SeO_3-0.4, Na_2WO_4-0.01) and vitamins (mg L^{-1}): biotin-0.02, nicotinamid-0.2, p-aminobenzoic acid-0.1, thiamin-0.2, panthotenic acid-0.1, pyridoxamine-0.5, cyanocobalamine-0.1, riboflavine-0.1. NaCl was added during ASM preparation at the concentrations tested for each experimental run, ranging from 16 to 150 g L^{-1} (1.6–15 % NaCl). The medium was autoclaved at 1.5 atm at 121 °C for 20 min. $Na_2S_2O_3$ was added after sterilization to the different concentrations tested (25 and 100 mM). The pH of the medium was adjusted to 7.0 ± 0.2 using 3 M NaOH stock solution after autoclavation or 50 mM PIPES [piperazine-N, N′-bis (2-ethanesulfonic acid) (Sigma Aldrich, Spain).

2.3. Experimental set-up

The experimental set-up consisted of three assays (Fig. 1). In the first test (TS1), strains initially identified through genomic mining were validated in the laboratory to confirm their ability to grow with CO_2 and $S_2O_3^{-2}$ as the sole energy and carbon source and assess their ectoine production capability. The second test (TS2) involved subjecting the

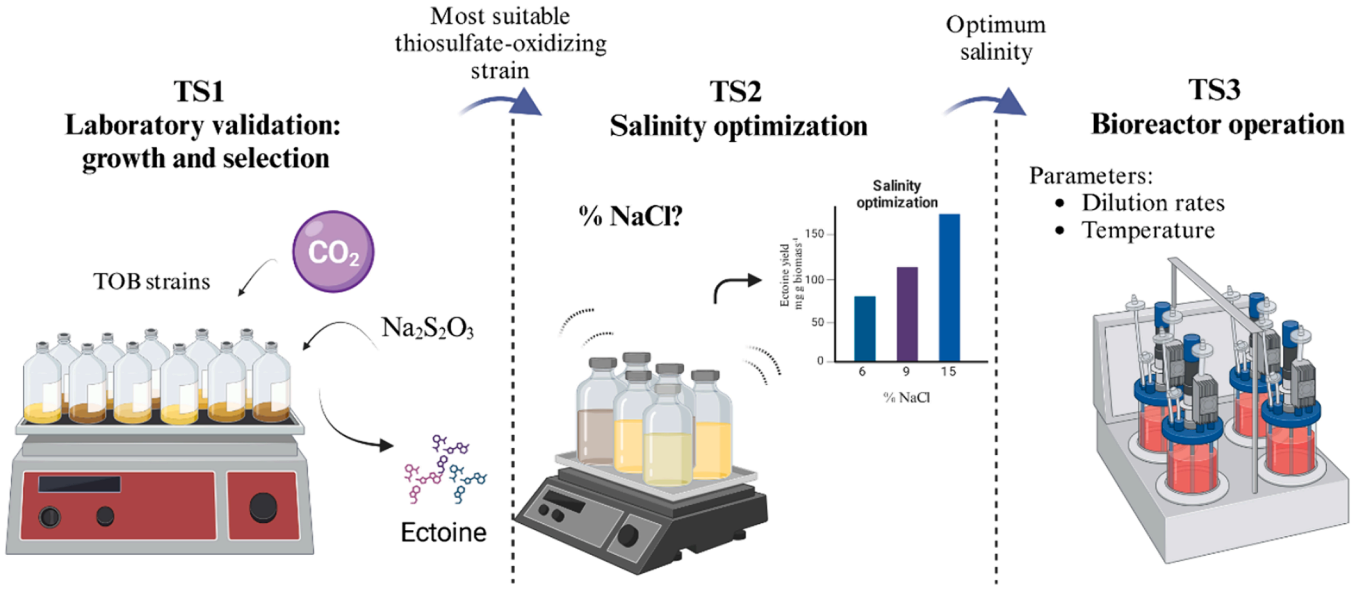

Fig. 1. Experimental set-up diagram.

most promising ectoine-producing strain to further optimization by testing it at several salinity concentrations to investigate the impact of salinity on both the growth and the ectoine production. Lastly, the third test (TS3), consisted of a bioconversion study in continuous bioreactors, where the optimal conditions determined in TS1 and TS2 were combined to validate ectoine production and contaminants depletion. Additionally, this process was optimized by examining the viability of ectoine production under lower and more cost-effective temperature conditions.

2.3.1. Test 1 (TS1). Laboratory validation: Growth and selection

Triplicate 250 mL glass bottles, each containing 100 mL of AMS medium adjusted at the optimal or lowest growth salinity for each strain with NaCl and optimum pH conditions (Table 1) were closed with gas-tight butyl septa and aluminum caps. CO_2 was then injected to the headspace at an initial concentration of 10 % (v/v) at 1 atm and the bottles were autoclaved. In the case of the bottles prepared for *Thiomicrospira aerophila*, 15 g L^{-1} of Na_2CO_3 and 20 g L^{-1} of $NaHCO_3$ were added instead of CO_2 after autoclavation. pH of the medium was adjusted using 3 M NaOH or 0.5 N of HCl stock solutions and 25 mM $Na_2S_2O_3$ were added after autoclavation from a stock solution of 1 M $Na_2S_2O_3$. The actively growing cultures identified as potential autotrophic thiosulfate-oxidizing strains were then inoculated at 5 % of inoculum. The cultures were incubated at 30 °C under orbital agitation at 200 rpm. To ensure biotic consumption, abiotic negative controls were prepared in the same manner. Periodically, 5 mL of liquid culture were withdrawn to assess biomass dry weight, doubling time and ectoine content, and this volume was replenished with 5 mL of fresh AMS medium. Monitorization of CO_2 concentration in the headspace was conducted to determine CO_2 consumption. The experiment lasted until no significant growth of the cultures was detected.

Table 1
Experimental growth conditions for thiosulfate-oxidizing strains in TS1.

Thiosulfate-oxidizing strains	CO_2/CO_3^{2-}	(% NaCl)	pH
Guyparkeria halophila DSM 6132	CO_2	3.0	7.0
Salinisphaera hydrothermalis DSM 21493	CO_2	2.5	5.5
Thiohalomonas denitrificans DSM 15841	CO_2	1.75	7.8
Thiomicrospira aerophila DSM 13739	$Na_2CO_3/NaHCO_3$	1.6, 3.0	9.8

2.3.2. Test 2 (TS2). Batch bioreactor operation for salinity optimization

TS2 was carried out to determine the effect of salinity in biomass growth and ectoine production. The most suitable strain, *G. halophila,* was selected and 3 different NaCl concentrations were tested: 6, 9, and 15 % of NaCl (60, 90 and 150 g L^{-1}). The experiment was conducted in 1 L gas-tight batch bioreactors, each of them containing 180 mL of sterilized AMS medium with 25 mM of $Na_2S_2O_3$ adjusted at the corresponding salinity. The headspace was replaced with an atmosphere containing 10:90 CO_2: air % (v/v). 10 mL of actively growing *G. halophila* were inoculated in each bottle. The bottles were incubated at the optimum growth temperature (30 °C) with an orbital agitation of 200 rpm. Each condition was assessed in triplicate. Abiotic negative controls were prepared in the same manner. Periodic assessments of CO_2 concentrations, biomass, and ectoine content were performed until no significant growth was observed.

2.3.3. Test 3 (TS3). Semi-continuous bioreactor operation

With the aim of mimicking the production of ectoine in a process similar to the one implemented in the ectoine production industry with heterotrophs, semi-continuous bioreactors were operated. For this, two different operational parameters were assessed in TS3: dilution rate (0.08, 0.04 and 0.02 d^{-1}) and temperature (25 and 30 °C). These temperatures were chosen based on the optimum growth temperature (30 °C) and the lowest growth temperature of the strain (25 °C), which is preferred from an economic perspective. For this, sterile gas-tight reactors of 2.1 L of capacity were filled with 380 mL of AMS at 150 g L^{-1} of NaCl and an atmosphere containing 10:90 CO_2: air % (v/v). In this occasion, 100 mM of $Na_2S_2O_3$ was used to avoid energy restrictive conditions. The reactors were inoculated with 15 mL of actively growing cultures of *G. halophila*. All reactors were operated under continuous agitation at 200 rpm. In total, six stages of 5-days (TS3-S1 to S6) were conducted in triplicate to evaluate the influence of the different operational parameters on biomass growth and ectoine production by *G. halophila* at the optimum salinity conditions (150 g L^{-1} NaCl) (Table 2). To ensure biological gas consumption, abiotic reactors as negative controls were prepared and operated in the same manner. O_2 was added to the headspace and its concentration maintained at 20 % (v/v) from TS3-S3 to avoid growth limitation. To prevent rapid acidification due to $S_2O_3^{-2}$ consumption, and subsequent biomass inhibition, pH was maintained during the experiment with 50 mM PIPES, pH 7 (Sigma Aldrich, Spain).

Table 2
Operational parameters assessed in TS3.

Test stage (S)	T (°C)	CO_2 gas-dissolved ($g{\cdot}m^{-3}$)	$S_2O_3^{-2}$ dissolved ($g{\cdot}m^{-3}$)	O_2 gas ($g{\cdot}m^{-3}$)	Dilution rate (d^{-1})
TS3-S1	30	298.7 ± 19.7	2.55E+04	127.3 ± 4.8	0.08
TS3-S2	25	306.0 ± 6.1	2.30E+04	133.9 ± 7.1	0.08
TS3-S3	30	236.0 ± 19.6	2.00E+04	250.6 ± 13.8	0.02
TS3-S4	25	249.0 ± 5.4	1.88E+04	268.1 ± 8.0	0.02
TS3-S5	30	247.7 ± 24.9	2.28E+04	247.2 ± 7.3	0.04
TS3-S6	25	292.5 ± 10.2	2.09E+04	271.9 ± 11.5	0.04

The withdrawn culture medium was utilized to determine the biomass dry weight, ectoine content, dissolved inorganic carbon (DIC), $S_2O_3^{-2}$ consumption and SO_4^{-2} production. CO_2 concentrations in the gas phase were monitored to determine CO_2 consumption.

2.4. Analytical methods

2.4.1. Ectoine determination

The intra-cellular ectoine contained in 4 mL of cultivation broth was extracted following the protocol described by Cantera et al. (2020). The concentration of ectoine was measured by two different HPLC methods and columns to ensure high quality quantification. For the first method, ectoine concentrations were detected and measured with a HPLC-UV in a 717 plus auto-sampler (Waters Alliance e2695, USA) coupled with a UV Dual λ Absorbance detector (Waters, USA) at 220 nm and 40 °C using a LC-18 AQ þ C Supelcosil column (Sigma Aldrich, Spain) and a C18 AQ + pre-column (Sigma Aldrich, Spain). A mobile phase of phosphate buffer, composed of 0.8 mM $K_2HPO_4{\cdot}3H_2O$ and 6.0 mM $Na_2HPO_4{\cdot}12H_2O$, was utilized at 25 °C with a flow rate of 1 mL min^{-1}. For the second quantification method, ectoine concentrations were detected and quantified by a HPLC LC_2050_C (Shimadzu, Japan) coupled with a UV Dual λ absorbance detector (Waters, USA) at 210 nm and 35 °C using an Spherisorb Amino (NH_2) Column (Waters, USA). A mobile phase of acetonitrile/H_2O, 75/25 (%) was used at a flow rate of 0.6 mL min^{-1}.

For ectoine quantification, external standards of commercially available ectoine [(S)-b-2-methyl-1,4,5,6-tetrahydro-pyrimidine-4-carboxylic acid, purity 95 %] (Sigma Aldrich, Spain) were employed. The specific intra-cellular ectoine content was calculated based on Eq. (1).

$$[Ect_{SP}]\left(mg_{Ect}\ g_{biomass}{}^{-1}\right) = \frac{mg_{\ intracellular\ ectoine}}{g_{dry\ weight\ biomass}} \quad (1)$$

2.4.2. Thiosulfate and sulfate quantification

$S_2O_3^{-2}$ and sulfate (SO_4^{-2}) concentrations were determined by HPLC-IC (Waters, Bellefonte, USA) coupled with a Waters 432 ionic conductivity detector and equipped with an IC-Pak Anion HC (150 mm × 4.6 mm) column (Waters, Bellefonte, USA). Periodically, 1 mL of each reactors culture was filtered through a 0.22 μm filter, transferred into HPLC vials and frozen until analysis. $S_2O_3^{-2}$ and SO_4^{-2} concentrations were quantified by preparing standard reference solutions with concentrations from 1.0 to 25.0 g L^{-1}. Sulfur balance was calculated according to Eq. (2) (Houghton et al., 2016):

$$S_2O_3{}^{-2} + 2\,O_{2(aq)} + H_2O \rightarrow 2\,SO_4{}^{-2} + 2\,H^+ \quad (2)$$

2.4.3. Carbon dioxide monitorization

CO_2 concentration was measured in an Agilent 7890A GC-TCD (Agilent technologies, USA), 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}. CO_2 consumption (Y) was calculated according to Eq. (3).

$$Y\left(\frac{CO_2}{X}\right) = \frac{-\Delta[CO2]_{e-phase}}{\Delta[X]_{e-phase}} \quad (3)$$

Where, $\Delta[CO_2]_{\text{e-phase}}$ is the variation in the concentration of CO_2 in the headspace and liquid phase during exponential growth phase and $[X]_{\text{e-phase}}$ is the variation in the concentration of biomass in the liquid phase during exponential growth phase.

CO_2 in the aqueous phase was calculated as the concentration of dissolved inorganic carbon (DIC). The aqueous CO_2 concentration was measured with a total organic carbon (TOC) analyser TOC-VCSH (Shimadzu, Japan). All samples were filtered through a 0.22 μm membrane and adjusted to pH 9 with NaOH 0.5 M prior to TOC measurement. The total CO_2 content was calculated as the sum of CO_2 in the gas and aqueous phase.

2.4.4. Biomass determination

Optical absorbance measurements at 600 nm (OD600) were conducted using a SPECTROstar Nano at 600 nm (BMG LABTECH, Germany). Dry biomass concentration was calculated as total suspended solids (TSS) according to Standard Methods (Baird and Bridgewater, 2017). The doubling time was calculated according to Eq. (4):

$$G = \ln(2)*\frac{(t2-t1)}{\left(\ln\left(\frac{i2}{i1}\right)\right)} \quad (4)$$

where, G is the generation time; t_1 and t_2, time 1 and time 2; i_1 and i_2, OD600 of the culture at time 1 and time 2.

The pH of the medium was determined using a SensIONTM + PH3 pHmeter (HACH, Spain).

3. Results and discussion

3.1. Selection of potential thiosulfate-oxidizing bacteria for ectoine production

Several chemolithotrophic thiosulfate-oxidizing bacteria strains were found from public genomic databases (NCBI) with the capacity to grow using CO_2 and $S_2O_3^{-2}$. In total, thirty-eight bacterial strains were found to be halophilic/halotolerant (see Supplementary materials 2). From those strains, seventeen were found to be aerobic and twenty-one anaerobic. Anaerobic strains were not subjected for further genomic analysis due to their requirement for an additional inorganic molecule, such as H_2, as electron donor when utilizing $S_2O_3^{-2}$ as electron acceptor. Additionally, anaerobic strains typically show lower biomass yields and slower growth rates (Bae et al., 2022). Moreover, anaerobic halophilic bacteria typically accumulate inorganic ions (K^+, Mg^{2+}, Na^+) to cope with external osmotic stress induced by elevated salt concentrations, rather than relying on compatible solutes (osmolytes) (Wang et al., 2023).

The genomes of the seventeen identified aerobic strains underwent screening to identify genes responsible for synthesizing ectoine. The ectoine synthesis pathway belongs to the aspartate family metabolism branch. This pathway comprises the sequential activity of the enzymes EctA, EctB, and EctC. In some microorganisms, ectoine can be further transformed to hydroxyectoine by a hydroxylase (EctD) (Chen et al., 2023). Additionally, the screening included checking for the existence of the gene *ectR*, a crucial transcriptional regulator in this pathway, as well as evaluating two genes responsible for generating the precursor L-2,4-diaminobutyrate: the genes for aspartate kinase (*ask*) and aspartate semi-aldehyde dehydrogenase (*asd*).

This genomic analysis resulted in the identification of six aerobic halophilic strains with the genomic potential to grow with CO_2 as the sole carbon source, $S_2O_3^{-2}$ as electron donor, and O_2 as electron acceptor, coupled with the ability to produce ectoines: *Guyparkeria halophila,*

Halothiobacillus neapolitanus, Salinisphaera hydrothermalis, Thiohalomonas denitrificans, Thiomicrospira aerophila and *Thiomicrospira halophila* (Fig. 2). Thus, making them potential candidates for the valorization of CO_2 and $S_2O_3^{-2}$ into ectoines.

3.2. TS1: laboratory screening for chemoautotrophic growth verification and ectoine production

In TS1, the initially identified strains were validated in the laboratory in order to verify an effective capacity to grow with CO_2 and $S_2O_3^{-2}$, as well as their capability to produce ectoine. Out of the total six strains previously identified, two of them (*Halothiobacillus neapolitanus* and *Thiomicrospira halophila*) were excluded from further analysis as they were not accessible in microbial public collections. The remaining strains, *Salinisphaera hydrothermalis*, *Thiohalomonas denitrificans, Thiomicrospira aerophila* and *Guyparkeria halophila*, were grown at their optimal or lowest growth salinity. Results of the average triplicates are summarized in Fig. 3. All the tested strains exhibited positive growth in the presence of CO_2 and $S_2O_3^{-2}$ concomitant to ectoine production.

Salinisphaera hydrothermalis has been characterized as a facultative autotrophic bacterium capable of fixing CO_2 using the Calvin-Besson cycle with $S_2O_3^{-2}$ as electron donor (Crespo-Medina et al., 2009). Ectoine was detected during growth with 2.5 % NaCl, reaching maximum values of 4.52 ± 2.6 $mg_{Ect}\ g_{biomass}^{-1}$ (0.5 % ectoine content). Moreover, the bacterium exhibited very long doubling times (345.8 ± 20.6 h) at the low tested salinity, suggesting a narrow halotolerance. Hence, *S. hydrothermalis* has limited potential for further implementation as a viable cell platform for ectoine production in the biotechnological industry.

Thiohalomonas species have been characterized as facultative anaerobic bacteria that can utilize reduced sulfur compounds as electron donors both aerobically (under micro-oxic conditions) or anaerobically (using nitrate as electron acceptor) (Sorokin et al., 2007). As a member of these genus, *Thiohalomonas denitrificans*, an obligated chemolithotroph bacterium, showed ectoine contents of 26.73 ± 9.34 $mg_{Ect}\ g_{biomass}^{-1}$ (3 % ectoine content) when cultivated with 1.75 % of NaCl under micro-oxic conditions. This ectoine content was relatively high in comparison to other ectoine producers at the low salinity conditions tested (Saum and Müller, 2008). In this sense, applying micro-oxic conditions for strain cultivation could potentially enhance cost-effectiveness by removing the need of intensive aeration systems. However, this strain exhibited the longest doubling time compared with all the strains examined (366.1 ± 11.5 h). These findings aligned with expectations, as previous studies have indicated that ectoine production usually relies in oxygen (Liu et al., 2021), therefore, under this condition where oxygen is limited or absent, the energy required for osmolyte production, such as ectoine, might not have been sufficient. This limitation could have hindered bacterial growth, leading to increased doubling times in micro-oxic conditions.

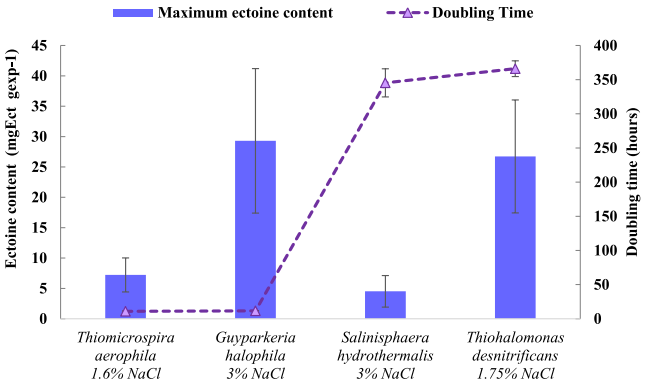

Fig. 3. Maximum ectoine contents (purple bars) and doubling times (dash line) for each of the thiosulfate-oxidizing strains tested in T1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Thiomicrospira aerophila is reported as an alkaliphilic, halotolerant chemolithoautotrophic sulfur-oxidizing bacterium capable to oxidize $S_2O_3^{-2}$ and sulfite (SO_3^{-2}) (Ang et al., 2017). In this study, *T. aerophila* displayed the shortest doubling time (10.9 ± 0.6 h), affirming its ability to grow rapidly in fully aerobic conditions (Sorokin et al., 2001). Despite demonstrating the capacity to produce ectoine, the content was remarkably low when cultivated with 1.6 % of NaCl (7.22 ± 2.8 $mg_{Ect}\ g_{biomass}^{-1}$, 0.7 % ectoine content). Subsequent tests were undertaken to assess whether *T. aerophila* could achieve higher ectoine contents by repeating the experiment with 3 % of NaCl. However, although previous studies indicated that this strain could grow with from 0.2-1.2 M of Na^+ with abundance of sodium carbonates ($NaCO_2$/$NaHCO_3$) (Banciu et al.,

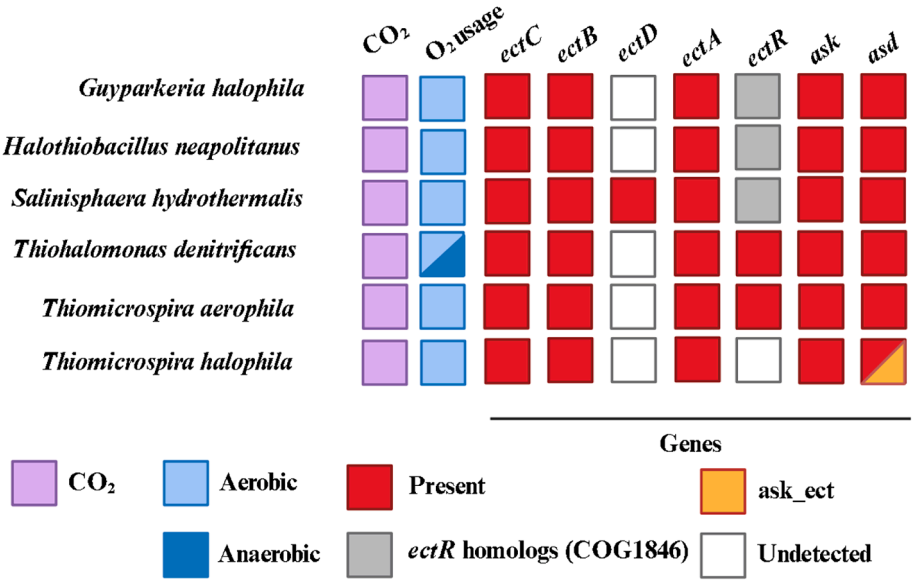

Fig. 2. Presence/absence of ectoine biosynthesis genes in halophilic/halotolerant chemolithoautotrophs that use S_2O3^{-2} as energy .
Source. ectC, ectoine synthase (EctC); ectB, DABA aminotransferase (EctB); ectD, ectoine hydroxylase (EctD); ectA, diaminobutyric acid (DABA) acetyltransferase (EctA); ectR, MarR-type regulator; ask, aspartate kinase (Ask); ask_ect, a specialized aspartate kinase (Ask); asd, l-aspartate-semialdehyde-dehydrogenase (Asd)

2005), in this study, negligible growth was detected for *T. aerophila* growing at 3 % of NaCl with sodium carbonates (0.6 M total Na^+). Consequently, due to this limitation, *T. aerophila* was not selected for further optimization.

Guyparkeria halophila is an obligate autotroph known for its capability to assimilate carbon through fixation using the Calvin-Benson cycle producing ribulose-1,5-bisphosphate (RuBP). This molecule undergoes a series of reactions in the glyoxylate cycle, where L-glutamine is formed. Following this, L-aspartate is synthesized through the metabolism of alanine, aspartate, and glutamine. This metabolic pathway subsequently produces L-2,4-diaminobutyrate, a precursor essential for the ectoine biosynthesis pathway (EctABC) (Lau Vetter et al., 2022). *Guyparkeria halophila* is also classified as an obligate aerobic, halophilic chemolithotroph with the capacity to oxidize various reduced sulfur compounds, including $S_2O_3^{-2}$, SO_3^{-2}, tetrathionate (S_4O^{-2}), or elemental sulfur (S_0) (Boden, 2017). In this study, *G. halophila* achieved growth with doubling times less than 12 h and exhibited the highest maximum ectoine content at 3 % NaCl, the minimum salinity required for its growth (29.3 ± 11.9 $mg_{Ect}\ g^{-1}_{biomass}$, 3 % ectoine content). As a result, *G. halophila* was selected as the most promising chemoautotrophic thiosulfate-oxidizing bacteria for further optimization analysis.

3.3. Salinity optimization for enhance ectoine production

Different salinity concentrations (6, 9, 15 % of NaCl) were tested to determine the effect of salt on growth and ectoine accumulation in the most promising ectoine producer thiosulfate-oxidizing bacterium, *G. halophila*. In all cases, *G. halophila* exhibited the highest ectoine content during the early exponential phase (47.6 ± 11.0, 91.9 ± 19.1 and 140.6 ± 18.0 $mg_{Ect}\ g^{-1}_{biomass}$ for 6, 9 and 15 % of NaCl, respectively), due to the initial hyperosmotic shock (Fig. 4). The transcription of ectoine biosynthetic gene clusters is typically strongly up-regulated when cells experience sudden high osmolarity (Hermann et al., 2020).

Ectoine content decreased at all salinities tested after 20 h of experiment. This gradual decrease could be attributed to the re-assimilation for cell metabolism, facilitated by the activity of ectoine degradation genes during the growth-retardation phase as carbon, nitrogen and energy reservoir (Czech et al., 2018). Genomic analysis confirmed that *Guyparkeria* poses *doe* genes (ectoine hydrolases) which metabolize ectoine to regenerate aspartate (Lau Vetter et al., 2022; Ngoc Pham et al., 2023).

G. halophila accumulated maximum ectoine contents of 4.8 % and 9.2 % of the total dry weight with 6 % and 9 % of NaCl, but this amount was almost doubled when the strain was grown under 15 % of NaCl, reaching ectoine contents up to 14.1 %. *G. halophila* has been reported to cope with a broad spectrum of salinities (from 3.0 – 23.2 % NaCl) (Supplementary materials 1). However, high-salt mediums pose challenges for further reactor implementation, such as the corrosion of steel fermenters and low gas solubility, resulting in the requirement of specialized materials and higher energy inputs (Liu et al., 2021). Hence, experiments involving salinities exceeding 15 % of NaCl would decrease the cost-effectiveness of these cell bioprospective platforms for ectoine production from gases.

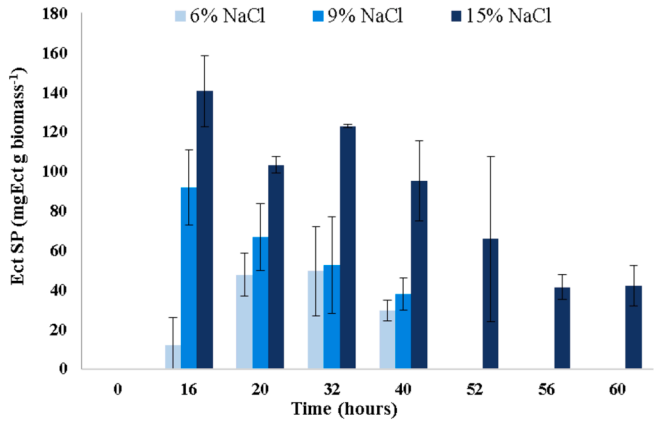

Fig. 4. Average ectoine contents during TS2 at the different salinities tested over time, light blue, 6% NaCl; blue, 9 % NaCl; dark blue, 15 % NaCl. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Salinity did not negatively affect growth, as the biomass values obtained were similar at 6 % and 15 % NaCl (90.0 ± 10.2, 58 ± 4.1 and 82.1 ± 10.1 mg L^{-1}, at 6, 9 and 15 %, respectively). Nevertheless, growth stopped when the pH reached values of 5.5 ± 0.6 at time 40 h in the case of the strain growing at 6 % and 9 % NaCl, and at time 60 h at 15 % NaCl. Pangenomic analysis revealed that *G. halophila* poses the complete sox pathway for $S_2O_3^{-2}$ oxidation (Lau Vetter et al., 2022). The completion of this pathway results in SO_4^{-2} as the sole product, leading to the release of protons by a hydrolysis from SoxB and subsequently explaining the observed decrease in pH (Zhang et al., 2020). In regard to these results, optimization of the salinities resulted in 15 % of NaCl as the optimum salinity for ectoine production of *G. halophila.* However, pH control is mandatory to maintain growth over time and stable ectoine and biomass values.

3.4. Batch bioconversion: ectoine production and carbon dioxide and thiosulfate depletion over time

In order to optimize the operational strategies for ectoine production and CO_2 removal with *G. halophila,* semi-continuous bioreactors at controlled pH were operated (Table 3; Supplementary materials 3).

During the operational stages TS3-S1 and TS3-S2, there was a discernible maximum consumption of CO_2 (1.8 ± 0.2 and 1.6 ± 0.5 $g_{CO2}\ g^{-1}_{biomass}$, respectively) and $S_2O_3^{-2}$ (48.8 ± 18.4 and 70.6 ± 23.3 $g^{-2}_{S2O3}\ g^{-1}_{biomass}$, respectively). SO_4^{-2} production was also detected in the medium, at concentrations of 16.9 ± 2.6 and 18.3 ± 8.1 $g^{-2}_{SO4}\ g^{-1}_{biomass}$, respectively. However, the significant loss of the total amount of sulfur in the system suggested the production of additional unaccounted sulphur products. Conversely, abiotic controls demonstrated no alteration in $S_2O_3^{-2}$ or SO_4^{-2} concentrations at both 30 and 25 °C. It is possible that *G. halophila* was generating unidentified sulphur species, such as tetrathionate, or elemental sulphur (S_0), as previous research encountered similar challenges with sulphur balance calculations in *Thiomicrospira crunogena* or *Thiobacillus* species (Houghton et al., 2016). Under oxygen-limited conditions, *G. halophila* may exhibit an incomplete thiosulfate oxidation pathway. Notably, rapid oxygen consumption by *G. halophila* was observed during both stages, resulting in oxygen concentrations in the headspace below 10 % (v/v). Therefore, the observed sulphur loss in the system may be attributed to the production of partially oxidized forms of sulphur or the growth of cells encapsulated in S_0, likely resulting from limitations in O_2 availability.

The highest ectoine contents were observed at 30 °C in TS3-S1 reaching almost 50 % of the total dry weight. At 25 °C, in TS3-S2, high concentrations were also detected (34.8 %), although not as prominently as under the optimum growth conditions. Both, in TS3-S1 and S2, a high dilution rate consistently kept cells in the early-exponential phase, leading to exponential ectoine production. This phenomenon can be attributed to the expression of ectoine biosynthesis genes, whose levels decrease as biomass growth stabilizes (Hermann et al., 2020). Nevertheless, the high dilution rates applied also led to the washout of biomass and a consistent low average biomass content was found in TS3-S1 and S2.

During TS3-S3 and TS3-S4 the dilution rate was decreased to 0.02 d^{-1} with the aim of avoiding biomass washout, while O_2 concentrations were maintained at 20 % (v/v) throughout the entire stages. Nevertheless, this promoted a significant decay in both CO_2 and $S_2O_3^{-2}$ consumption. At this low dilution rate, rapid proton production during $S_2O_3^{-2}$ oxidation caused a pH drop, reaching levels as low as 5.5 in TS3-

Table 3
Average values of each experimental test obtained in TS3.

Test stage (S)	T°(°C)	Dilution rate (d^{-1})	$Y\ CO_2$ ($g_{CO2}\ g^{-1}_{biomass}$)	$Y\ S_2O_3^{-2}$ ($g^{-2}_{S2O3}\ g^{-1}_{biomass}$)	$P\ SO_4^{2-}$ ($g^{-2}_{SO4}\ g^{-1}_{biomass}$)	TSS ($mg\ L^{-1}$)	Ectoine content ($mg_{Ect}\ g^{-1}_{biomass}$)
TS3-S1	30	0.08	1.8 ± 0.2^{a}	48.8 ± 18.4^{a}	16.9 ± 2.6^{a}	10.3 ± 3.1^{a}	476.9 ± 37.1^{a}
TS3-S2	25	0.08	1.6 ± 0.5^{a}	70.6 ± 23.3^{a}	18.3 ± 8.1^{a}	12.6 ± 4.4^{a}	$348.1 \pm 70.0^{a,c}$
TS3-S3	30	0.02	0.2 ± 0.1^{b}	20.2 ± 9.6^{b}	2.4 ± 1.5^{b}	10.5 ± 3.6^{a}	147.6 ± 74.9^{b}
TS3-S4	25	0.02	$0.5 \pm 0.2^{b,c}$	$36.8 \pm 17.3^{b,c}$	13.1 ± 6.3^{a}	15.3 ± 6.2^{a}	$217.1 \pm 15.8^{b,c}$
TS3-S5	30	0.04	$0.7 \pm 0.3^{b,c}$	$25.4 \pm 5.1^{b,c}$	2.2 ± 0.9^{b}	24.3 ± 8.2^{b}	250.6 ± 59.7^{c}
TS3-S6	25	0.04	0.6 ± 0.1^{c}	52.6 ± 18.9^{c}	$43.6 \pm 26.3^{a,c}$	20.5 ± 5.1^{b}	$217.1 \pm 7.3^{b,c}$

Y = consumption, P = production. Values with different letters for each parameter are statistically different at level 0.05. Values are means ± SD of triplicate measurements.

S3 and 6.5 in TS3-S4 despite the addition of PIPES buffer during the experiment, thereby, inhibiting biomass growth and promoting S_0 precipitation. Concurrently, ectoine contents exhibited a notable decrease to 14.7 % in TS3-S3 and 21.7 % in TS3-S4. Thus, the adoption of a lower dilution rate failed to stimulate biomass proliferation and, consequently, ectoine production. This effect was more pronounced at 30 °C, where the bacteria metabolized $S_2O_3^{-2}$ more rapidly, resulting in a greater pH decrease.

To avoid the negative effect of too low or high dilution rates, last tests (TS3-S5 and S6) were conducted at 0.04 d^{-1}. Although biomass content increased to 24.3 ± 8.2 mg L^{-1} and to 20.5 ± 5.1 mg L^{-1} at 30 and 25 °C, CO_2 consumption did not improve in comparison to TS3-S1 and TS3-S2. This most likely was the result of minimizing cell washout and favored "old" cells accumulation which led to the reduction in substrates consumption. Concomitantly, ectoine content diminished to 25 % and 21.7 % in comparison to TS3-S1 and S2 where almost doubled ectoine contents were achieved. This was most likely due to the reutilization of ectoine from the previously synthesized reservoir and a decline in the expression of ectoine genes resulting from metabolic adaptation.

Our findings indicate that the most effective operational approach in semi-continuous bioreactors involves utilizing the strain's optimal growth temperature alongside high dilution rates to achieve maximum ectoine productivities and CO_2 removal rates. Oxygen needs to be controlled and maintained at 20 % (v/v) to promote the complete oxidation of $S_2O_3^{-2}$ to SO_4^{2-}. Remarkably, the maximum ectoine contents obtained at 15 % NaCl during semi-continuous operation in TS3 were significantly higher than the ones found in TS2 (14 %). This result could be correlated with the higher amount of energy ($S_2O_3^{-2}$) available for the bacteria (100 mM of $Na_2S_2O_3$), pH control, and to the higher dilution rates with the removal of sulphur metabolites.

The maximum specific ectoine content obtained with *G. halophila* in this study stands out as one of the highest reported among natural halophilic ectoine producers, however, titters and yields are still far from those achieved by industrial microorganisms, including GMOs fed with sugars. This is particularly advantageous given the utilization of residual industrial compounds and the prevalent greenhouse gas, CO_2, for ectoine production. In this study, maximum specific ectoine contents reached almost 50 % of the total dry weight. These results are comparable with ectoine contents reported with *Halomonas salina* DSM 5928 with 0.5 M of NaCl but using sodium glutamate as substrate (Zhang et al., 2009), or *Chromohalobacter salexigens* DSM 3043 at 1.8 M of NaCl, using glucose as substrate (Fallet et al., 2010).

Research focused on Genetically Modified Organisms (GMOs) for ectoine production reported higher ectoine contents always using heterotrophic organisms (Gießelmann et al., 2019; He et al., 2015). However, the use of GMOs for ectoine production demonstrate several challenges including the unsteadiness of GMOs as ectoine producers, along with the limited appeal that GMOs hold in the cosmetic, pharmaceutical, and medical markets (Becker and Wittmann, 2020).

4. Conclusions

Results from this study demonstrated that *G. halophila* reached maximum ectoine contents up to 50 % (473.9 ± 37.1 mg of ectoine g $biomass^{-1}$). These results not only constitute the highest ectoine contents so far reported by autotrophs and most of heterotrophs, but also, the first proof of a novel valorization platform for CO_2 and $S_2O_3^{-2}$, establishing a foundation for a new economic niche focused on transforming CO_2 into pharmaceuticals.

CRediT authorship contribution statement

E. Huang-Lin: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **D. Tamarit:** Software, Investigation, Formal analysis, Data curation. **R. Lebrero:** Writing – review & editing, Validation, Supervision. **S. Cantera:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.131152.

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