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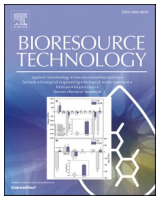

Microbial conversion of carbon dioxide and hydrogen into the fine chemicals hydroxyectoine and ectoine

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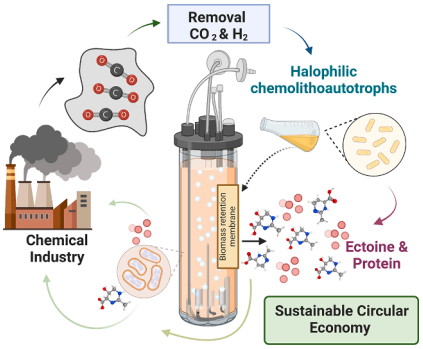
HIGHLIGHTS

- A sustainable biotechnology targeting to de-fossilize the pharmaceutical industry.
- CO_2 and H_2 are transformed with unexplored bacteria into chemicals used in medicine.
- New cell platforms were found using genomic mining and validated in the laboratory.
- Ectoine contents up to 9% and hydroxyectoine contents up to 6% were obtained.
- First proof of concept of a novel biotechnology to valorize CO_2 into ectoines.

GRAPHICAL ABSTRACT



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ABSTRACT

This study explores a novel conversion of CO_2 into the chemicals hydroxyectoine and ectoine, which are compounds with high retail values in the pharmaceutical industry. Firstly, 11 species of microbes able to use CO_2 and H_2 and that have the genes for ectoines synthesis (*ectABCD*) were identified through literature search and genomic mining. Laboratory tests were then conducted to ascertain the capacity of these microbes to produce ectoines from CO_2. Results showed that the most promising bacteria for CO_2 to ectoines bioconversion are *Hydrogenovibrio marinus, Rhodococcus opacus,* and *Hydrogenibacillus schlegelii.* Upon salinity and $H_2/CO_2/O_2$ ratio optimization, *H. marinus* accumulated 85 mg of ectoine g biomass^{-1}. Interestingly, *R. opacus* and *H. schlegelii* mainly produced hydroxyectoine (53 and 62 mg g biomass^{-1}), which has a higher commercial value. Overall, these results constitute the first proof of a novel valorization platform of CO_2 and lay the foundation for a new economic niche aimed at CO_2 recircularization into pharmaceuticals.

1. Introduction

The utilization of carbon dioxide (CO_2) as a carbon block for the synthesis of valuable chemicals and fuels is of increasing interest (Kumar et al., 2018). CO_2 emissions represent the biggest fraction (78.8 %, data from 2021) of the total emitted greenhouse gases (GHG) worldwide (EEA (European Environmental Agency), 2021; National Oceanic and Atmospheric Administration, NOAA, 2020). A large part of these

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emissions is vented into the atmosphere by means of industrial processes. Industrially produced CO_2 can be emitted in the form of highly concentrated flue gas streams (15–80 % CO_2), e.g. in industrial processes producing ammonia, ethanol, steel, and paper or in the form of lower concentration emissions (8–10 % CO_2), such as in natural gas-fired power plants or biogas combustion (Kumar et al., 2018). In these scenarios, CO_2 is a perfect feedstock for in situ transformation into chemicals using autotrophic organisms without light requirements (dark fixation) (Nisar et al., 2021).

Current dark CO_2 fixation processes target relatively low value bulk chemicals, such as alcohols, fatty acids, and organic acids, and are based on the application of a restricted number of model organisms (Kumar et al., 2018). Thus, the expansion of both, the variety of products generated from CO_2 (e.g. chemicals for cosmetics and pharmaceuticals) and the type of autotrophic organisms used for CO_2 conversion, is interesting to broaden the potential of dark CO_2 fixation processes with application in diverse industries.

Interesting products, due to their high value, are those produced by extremophilic bacteria and archaea that live at high salinity – specifically the extremolyte, ectoine, and its derivate, hydroxyectoine (Becker and Wittmann, 2020; Widderich et al., 2016). Ectoine accounts for a retail value of €1000 kg^{-1} and has exceptional properties (protectors of cell membranes and tissues) and is implemented in health-promoting and therapeutic activities (Liu et al., 2019). Hydroxyectoine, due to its hydroxylated nature, confers additional protective properties against heat and dryness and it has an even higher commercial value than ectoine (Liu et al., 2019). Both, ectoine and hydroxyectoine (ectoines), are manufactured through bioprocesses using sugars. However, little research has explored the possibility of the production of ectoines from renewable or waste-based carbon sources (Cantera et al., 2018; Kunte et al., 2014). In fact, the transformation of CO_2 into these compounds has never been experimentally assessed.

One of the main difficulties for the cost-effective microbial transformation of CO_2 is the selection of a suitable, available energy source. Chemolithoautotrophs can use a variety of energy sources – from metals and reduced sulfur and nitrogen compounds, to gaseous compounds such as, CO or H_2 (Anand et al., 2020). Although the use of liquid waste streams containing any of the potential electron donors can be of great interest from an economic point of view, manufacturing products suitable for human-use requires a feedstock that is free of potential hazardous materials (Kunte et al., 2014). In this regard, the use of clean gases as an energy source, such as H_2 or impurity free pre-treated syngas, can be the best approach for the production of chemicals for pharmaceuticals, cosmetics, and medicines. Renewable H_2 can be produced via a number of sustainable technologies, including biomass pyrolysis-gasification, biomass dark fermentation, supercritical water gasification, and water electrolysis using renewable electricity (Bommareddy et al., 2020).

Usually, the organisms implemented for the transformation of CO_2 and H_2 are anaerobic chemolithoautotrophs, mainly acetogens, which suffer from energetic limitations with final low productivities of the chemical (Katsyv and Müller, 2020). Conversely, aerobic chemolithoautotrophs – microorganisms able to grow with CO_2 as the sole carbon source and H_2 as the energy source, in the presence of air – could become a cost-effective, sustainable, circular and climate neutral novel platform for the production of pharmaceuticals.

To this aim, this research identified novel aerobic microbes able to produce ectoines from CO_2 and H_2. First, halophilic microbes able to use CO_2 and H_2 as the carbon or energy source were determined based on literature and databases and their genomes downloaded and mined for the genes involved in ectoine and hydroxyectoine synthesis pathways. The transformation of CO_2 into ectoines was tested in the laboratory for proof-of-concept validation. The most promising bacteria, according to their growth rates and (hydroxy)ectoine production, were implemented in batch bioreactors and the process was optimized to obtain high (hydroxy)ectoine content linked to CO_2 removal.

2. Materials and Methods

2.1. Selection of potential chemolithoautotrophic organisms able to produce ectoine

Halophilic and halotolerant chemolithotrophs were identified from literature searches (see supplementary material). Genomes of the identified species were downloaded from the National Center for Biotechnology Information (NCBI, Datasets v. 10.0.0; (see supplementary material)) and screened for genes encoding for enzymes involved in ectoines' synthesis pathways, specifically: *ectA, ectB*, *ectC* and *ectD*. Moreover, the presence of *ectR*, an important transcriptional regulator of this pathway, and two genes involved in the production of the precursor L-2,4-diaminobutyrate (aspartate kinase: *ask* and aspartate semialdehyde dehydrogenase: *asd*), were also assessed. Alignments or profile hidden-Markov models (HMM) for each gene were retrieved from public databases (see supplementary material). HMMer 3.1b2 (https://hmmer.org) was used to build profile HMMs of gene alignments, and to search all HMMs against the downloaded proteomes. To corroborate the detection of gene orthologs, the protein sequences with significant hits ($<1e^{-5}$) were retrieved and annotated using Interproscan v.5.36–75.0 (Jones et al., 2014). First, protein sequences identified as EctB and EctC were selected if they yielded significant ($<1e^{-5}$) Interproscan hits to TIGR00709 and PF06339, respectively. Protein sequences detected as EctD and EctA were corroborated as such if they had significant Interproscan hits to PF05721 and PF00583, respectively; sequences detected as EctR were selected if a Blastp search against the COG database (Galperin et al., 2015) retrieved at least 5 significant (<1e-5) best hits against proteins classified as COG1846.

2.2. Source of microorganisms

All strains used in this study were purchased from DSMZ (Brunswick, Germany): *Alkalilimnicola ehrlichii* (DSM 17681), *Hydrogenibacillus schlegelii* (DSM 2000), *Hydrogenovibrio marinus* (DSM 11271), *Pseudonocardia autotrophica* (DSM 535), *Pseudonocardia carboxydivorans* (DSM 43559), *Pseudonocardia dioxanivorans* (DSM 44775) and *Rhodococcus opacus* (DSM 43205). Physiological characteristics of these strains are included in Table S1. All cultures were purchased as freeze dried biomass, except *H. marinus* that was purchased as an actively growing culture in medium (DSM 744). *H. schlegelii* was activated in medium DSM 260; all the other freeze dried cultures were activated in trypticase soy broth (medium DSM 535). Inoculation from freeze dried stocks was done in sealed serum bottles of 200 mL containing 100 mL of medium and 100 mL of air in the headspace at atmospheric pressure.

2.3. Mineral salt medium

Growth tests with H_2/CO_2 were performed using ammonium mineral salt medium (AMS). This medium contained (g L^{-1}): $MgSO_4{\cdot}7H_2O$ – 1.0, $CaCl_2{\cdot}2H_2O$ – 0.11, NH_4Cl_2 – 0.5, $FeSO_4{\bullet}7\ H_2O$ – 0.005. 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 to the AMS at different concentrations (from 30 to 100 g/L). Nitrate was added as KNO_3^- at a final concentration of 620.0 mg NO_3^- L^{-1}. Nitrate was added to the AMS to avoid nitrogen limitation during ectoine production and also to study its potential use as electron acceptor when there was oxygen limitation. AMS was autoclaved at 1.5 atm and 121°C for 20 min. pH was buffered around 7 by adding 15 mL L^{-1} of sterile stock solution of 50.0 mL L^{-1} of $NaHCO_3$ (1 M), 5.0 mL L^{-1} of Na_2CO_3 (1 M) and 20.0 mL L^{-1} of sterile stock solution of KH_2PO_4 (0.1 M) and $Na_2HPO_4{\bullet}12\ H_2O$ (0.08 M). Tests where CO_2 was monitored were not supplemented with

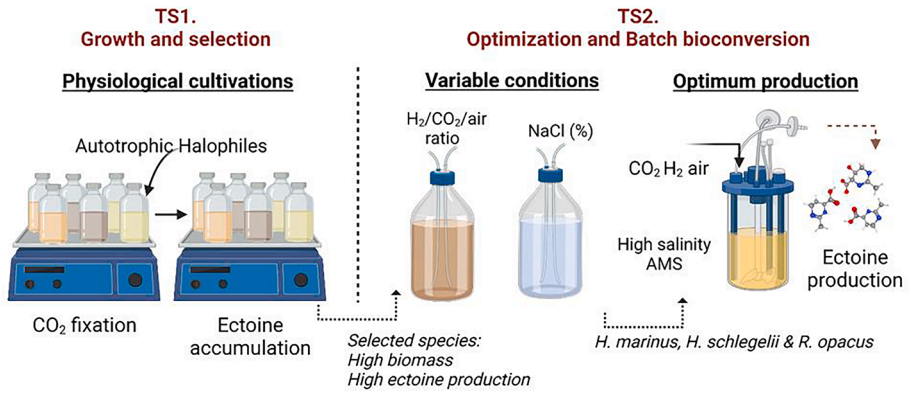

Fig. 1. Experimental setup of this study.

carbonate/bicarbonate buffer and initial pH was adjusted to 7 using a solution of NaOH 3 M.

2.4. Analytical procedures

2.4.1. Ectoine, hydroxyectoine and nitrate determination

The intra-cellular ectoine and hydroxyectoine in 2 mL of cultivation broth was extracted following the protocol described by Cantera et al. (2018). The concentration of ectoine was measured with a Shimadzu Prominence-i LC-2030C plus HPLC (Shimadzu, Japan) with a UV detector at 210 nm. HPLC was equipped with a Polaris, NH2, 180 Å, 3 μm, 4,6 × 150 mm (Agilent Technologies, USA). The mobile phase was acetonitrile/H_2O 75/25 (%) at a flow rate of 0.6 mL min^{-1}. Oven temperature was set at 30 °C. 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) were used for quantification. The intra-cellular ectoine and hydroxyectoine content were calculated according to Eq.1 (1).

$$\frac{Ect}{Hydro}content = \frac{[Ect - Hydro]}{[X]} \quad (1)$$

where [Ect-Hydro] is the ectoine or hydroxyectoine concentration and [X] is the biomass dry weight. For flocculating species (where non-representative liquid sample could be withdrawn), the Ect-Hydro content was obtained by collecting the total liquid of the bottles.

Nitrate from the supernatant was measured on a ICS-2100 (Thermo Scientific, USA) equipped with an AS19 column, 2x250 mm (Thermo Scientific, USA). The mobile phase consisted of a gradient of KOH, ranging from 1 to 40 mM KOH, generated by a cartridge and the flow was 0.4 mL min^{-1}. 30 μL of the supernatant were transferred to HPLC vials containing 970 μL of milli-Q water. The peaks were detected using a suppressed conductivity detector.

2.4.2. H_2 and CO_2 determination

CO_2 and H_2 were measured with a Compact GC equipped with 2 lines, both with a TCD detector (Interscience, The Netherlands). To measure CO_2 a RT-Q-bond column,10 m × 0.32 mm (Restek, USA) was used. The valve (injection) oven and the column oven were set at 100 °C, the TCD detector temperature was 110 °C and the TCD filament temperature was 175 °C. The carrier gas was Argon with an applied pressure of 80 kPa. For H_2, a Carboxen 1010 column,3 m × 0.32 mm (Sigma-Aldrich, USA) followed by a Molsieve 5A column,30 m × 0.32 mm (Sigma-Aldrich, USA) was used. The valve (injection), the column oven, the TCD detector temperature and the TCD filament temperature were set at 100, 140, 110 and 175 °C, respectively. Argon was again the carrier gas, here with an applied pressure of 325 kPa.

The CO_2 and H_2 consumption were calculated using Eq. (2).

$$Y_{CO2/X} = \frac{-\Delta[CO_2]_{e-phase}}{\Delta[X]_{e-phase}} \quad (2)$$

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

The total CO_2 content (g) was calculated as the sum of CO_2 in the gas and aqueous phases. The concentration (g m^{-3}) of CO_2 in the headspace at atmospheric pressure was determined with the GC-TCD. The aqueous CO_2 concentration was estimated according to the dimensionless Henry constant for CO_2 (KHcc = 1.22 (gas phase/liquid phase)). The amount of H_2CO_3 and CO_3^- were calculated using the Henderson-Hasselbach equation (Eq. (3)):

$$pH = pK_a + log_{10}\left(\frac{[HCO_3^-]}{[H_2CO_3]}\right) \quad (3)$$

$$[HCO_3^-] = [H_2CO_3]*10^{pH-pK_{a1}}$$

$$[CO_3^{2-}] = [HCO_3^-]*10^{pH-pK_{a2}}$$

where, pK_{a1} is the acid dissociation constant from H_2CO_3 (=6.35) and pK_{a2} is the basic dissociation constant from HCO_3^- (=10.33). The pH considered was the one obtained measuring at the time of gas sampling. pH was determined using a Thermo pH Ross semi-micro electrode. The gases were always measure at 20 °C.

2.4.3. Biomass determination

Optical absorbance at 600 nm (OD600) was measured using a UV/Vis spectrophotometer (Shimadzu Europa GmbH, Germany). Dry biomass concentration was measured as total suspended solids according to Standard Methods for the examination of water and wastewater (Baird and Bridgewater, 2017). The doubling time was calculated according to Eq. (4).

$$G = \ln(2)*\frac{t_2 - t_1}{\ln\left(\frac{i_2}{i_1}\right)} \quad (4)$$

where G is the generation time, t_1 and t_2, time 1 and time 2, i_1 and i_2 number of cells at time 1 and time 2.

2.5. Experimental setup

The experimental setup was divided in two assays (Fig. 1). Test Series 1 (TS1) consisted of an initial screening of the selected bacteria from genome mining to verify i) the utilization of CO_2 and H_2 as carbon and energy sources, and ii) their capability to produce ectoines (Fig. 1). In Test Series 2 (TS2), the most productive bacteria in TS1 were optimized in 1L gas-tight bioreactors under different conditions (H_2/O_2 ratios,

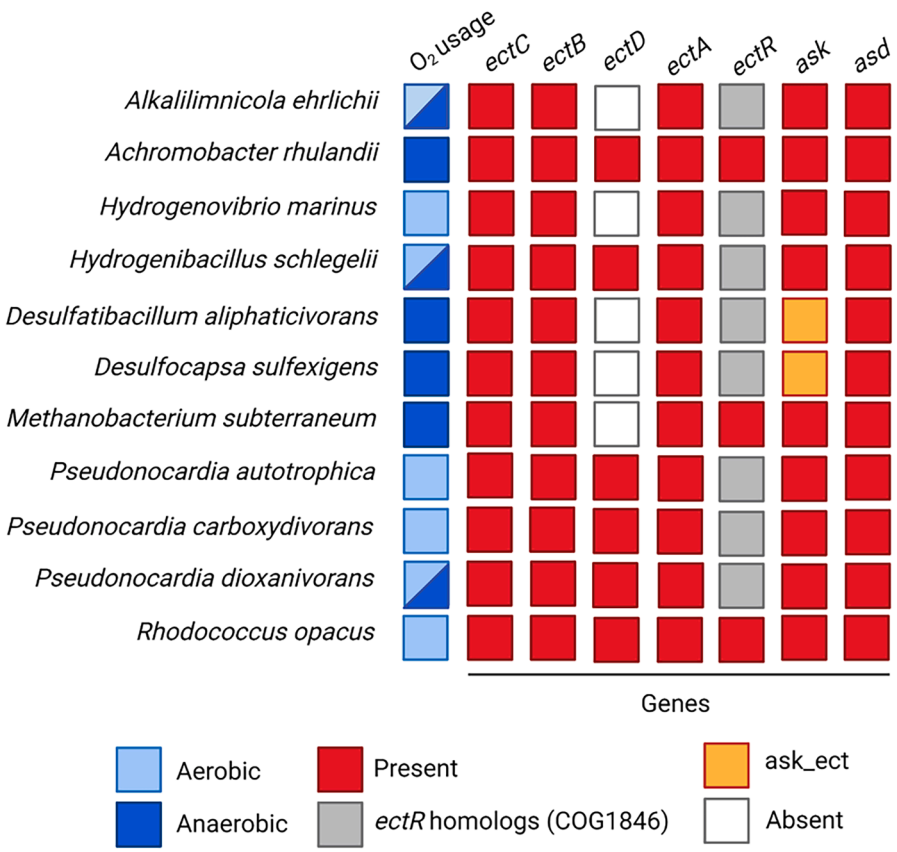

Fig. 2. Presence/absence of ectoine and hydroxyectoine biosynthesis genes in halophilic/halotolerant chemolithoautotrophs that use H_2 as energy source. *ectC*, ectoine synthase (EctC); *ectB*, DABA aminotransferase (EctB); *ectD*, ectoine hydroxylase (EctD); *ectA*, diaminobutyric acid (DABA) acetyltransferase (EctA); *ectR*, MarR-type regulator (*ectR* homologs are considered genes belonging to the *ectR* gene family found within 10 kb of the *ect* gene cluster); *ask*, aspartate kinase (Ask); *ask_ect*, a specialized aspartate kinase (Ask); *asd*, l-aspartate-semialdehyde-dehydrogenase (Asd).

salinity) to study the effect on growth and ectoine production.

2.5.1. Test series 1: Growth and selection

In TS1, 1 mL of exponentially grown cultures was inoculated in 200 mL glass bottles containing 100 mL of AMS supplemented with 3 % NaCl. The bottles were closed using gas-tight butyl septa and aluminum caps. CO_2, H_2 and air were then injected to the headspace at an initial concentration of 10 CO_2: 40 H_2: 50 air (% v/v, 1 atm) with a gas blender GB4000 of 4 channels (mcq instruments, Italy) connected to 3 gas lines containing H_2, CO_2 and air. The bottles were autoclaved and the buffer and vitamins added (section 2.2). Cultures were incubated at 30 °C, except for *H. schlegelli* that was incubated at 65 °C. Bottles were kept under orbital agitation at 200 rpm. The cultures were transferred three consecutive times in AMS under an atmosphere of 10 CO_2: 40 H_2: 50 air (% v/v, 1 atm) to remove any remaining carbon sources. Assays in TS1 were performed in quintuplicate (for each of the tested strains). Abiotic negative controls, prepared in the same way as test bottles but without addition of inoculum were also set-up. Biotic controls, prepared with trypticase soy broth, were used to ensure that cells were viable at the time of inoculation. H_2 and CO_2 concentration were monitored daily. For non-flocculating species (*H. schlegelii, H. marinus, R. opacus*), periodically, 5 mL of liquid culture were withdrawn to determine biomass dry weight, ectoine and hydroxyectoine content. This 5 mL were replaced with fresh AMS. The values obtained are represented as the average ± standard deviation of the quintuplicates. For flocculating species (*P. autotrophica, P. carboxydivorans* and *P. dioxanivorans*) a bottle from the quintuplicates was sacrificed at each sampling point to measure ectoine and hydroxyectoine content and biomass dry weight. Average biomass concentrations were not obtained in the flocculating species. The experiments lasted until H_2 complete depletion. An extra experiment was carried out to study the conversion of CO into ectoines. This experiment was developed as TS1, using syngas (10 CO_2: 30 H_2: 20 CO: 40 air, % v/v, 1 atm) and 80 CO: 20 O_2 (% v/v, 1 atm) in the headspace. H_2, CO and CO_2 concentration were monitored every-three days (gas sampling was less frequent due to the lower growth rate of the strains in CO).

2.5.2. Test series 2: Optimization of culture conditions

TS2 was performed to evaluate the influence of different environmental factors – H_2:O_2 ratio and concentration of NaCl – on the production of ectoine and hydroxyectoine by the bacterial species selected in TS1. Sterile batch gas-tight reactors (1.0 L) containing 190 mL of AMS were used in TS2 assays.

2.5.3 Effect of salinity and H_2:O_2 ratio

The effect of H_2:O_2 ratio was studied using 5 different ratios of these two gases: 70:30, 60:40, 50:50, 40:60 and 30:70 (H_2: air, respectively) with AMS at 3 % NaCl and at atmospheric pressure. The effect of salinity on the production of ectoines was tested at 8 different NaCl concentrations: 30, 40, 50, 60, 70, 80, 90 and 100 g/L. Salinity tests were performed under an atmosphere of 30: 70 H_2: air. Each condition was tested in triplicate. Carbonate and bicarbonate were used to maintain the pH at 7.0 ± 0.3. The batch reactors were inoculated with 10 mL of the inoculum from TS1 at an initial concentration of 7.1 ± 4.4 mg L^{-1} (Note that this value corresponds to the average initial concentration of the three selected strains). The headspace atmospheric pressure was maintained along time adding N_2 to the headspace. Gas, biomass and ectoine and hydroxyectoine concentrations were monitored daily until H_2 or oxygen depletion.

2.5.4 Bioconversion in the optimum conditions for each strain

The batch bioreactors were filled with 190 mL of AMS and a gas mixture of 30:10:60 H_2: CO_2: air, sealed and autoclaved in quadruplicate. NaOH 3 M was used to adjust the pH to initial values of 6.95 ± 0.1. Carbonate/bicarbonate buffer was omitted to monitor accurately the fixation of CO_2 along time. The experiment was conducted with AMS 5

% NaCl for *H. schlegelii*, AMS 6 % NaCl for *H. marinus* and AMS 7 % NaCl for *R. opacus*. The batch reactors were inoculated with a final biomass concentration of 12 ± 4.5 mg L^{-1}. Headspace pressure in bioreactors was maintained at 1 atm along time by adding air. Everyday 5 mL of AMS were withdrawn to measure ectoine, hydroxyectoine, biomass and pH. This 5 mL were replaced with fresh medium resulting in a dilution rate of 0.025 d^{-1}. The experiments lasted until complete H_2 depletion.

3. Results and discussion

3.1. Selection of potential chemolithoautotrophic ectoine-producing microorganisms

Several halophilic microbes have the ability to grow on CO_2 and H_2, but their potential to produce ectoines using these gases is unexplored. Based on literature and genomic databases, a total of sixty-two species of halophilic chemolithotrophs able to grow with CO_2 as sole carbon source and H_2 as the energy source were gathered (Table S1). Eleven of them were aerobic and fifty-one anaerobic. Thirty-six of them had their genomes available in public databases (Table S2).

The pathway for ectoine production involves three specific enzymes: l-2,4-Diaminobutyric acid (DABA) transaminase (EctB), DABA acetyltransferase (EctA) and ectoine synthase (EctC) enconded by the *ectABC* gene cluster(Czech et al., 2019). Some organisms also present the gen *ectD* that codifies for an ectoine hydroxylase (EctD), which is required to produce hydroxyectoine (Czech et al., 2018). Moreover, the enzymes aspartate kinase (Ask) and aspartate-semialdehyde-dehydrogenase (Asd) are necessary to produce the precursors for ectoine production. The genomic screening developed to identify species that had these genes showed that eleven of the genomes screened had the genes to codify the enzymes for ectoine synthesis and six for hydroxyectoine synthesis (Fig. 2).

Genomic analysis resulted in the identification of seven aerobic microorganisms with the genes to produce ectoine from CO_2/H_2: *Alkalilimnicola ehrlichii, Hydrogenovibrio marinus, Hydrogenibacillus schlegelii, Pseudonocardia autotrophica, Pseudonocardia carboxydivorans, Pseudonocardia dioxanivorans* and *Rhodococcus opacus*. Genomes of these microorganisms contain genes necessary to produce precursors for ectoine synthesis (aspartate kinase (*ask*) and aspartate semi-aldehyde dehydrogenase (*asd*)) and genes for ectoine production (*ectA*, *ectB* and *ectC*). Moreover, the species of *H. marinus*, *P. autotrophica, P.carboxydivorans* and *P. dioxinivorans* and *R. opacus* can potentially accumulate hydroxyectoine because they possess the gen *ectD* in their genomes.

On the other hand, only four anaerobic organisms; *Achromobacter ruhlandii, Desulfatibacillum aliphaticivorans, Desulfocapsa sulfexigens* and *Methanobacterium subterraneum* had the genes necessary to encode proteins for the formation of the ectoine precursor L-2,4-diaminobutyrate (*asd* and *ask*) and for ectoine synthesis (*ectA*, *ectB* and *ectC)*. *A. ruhlandii* also possesses the gene *ectD* to hydroxylate ectoine into hydroxyectoine.

3.2. Experimental screening for ectoine and hydroxyectoine producers

In TS1, aerobic strains identified in the genomic analysis (section 3.1) as potential ectoine and hydroxyectoine producers were tested in the lab for the effective capability to produce these compounds from CO_2/H_2. Anaerobic microorganisms were not tested due to their lower biomass yields and smaller growth rates that these organisms usually encounter (Bae et al., 2022). The results of the average values obtained in the replicates are summarized in table 1. *Alkalilimnicola ehrlichii* has been characterized as a facultative chemoautotrophic bacterium able to use different electron donors for CO_2 fixation (arsenite, hydrogen, sulfide or thiosulfate) (Hoeft et al., 2007). However, in this study, it did not show activity or growth on CO_2/H_2 (after three trials), despite viability of the culture when grown in rich medium. Several *Pseudonocardia* species are able to grow autotrophically (Grostern and Alvarez-Cohen, 2013). From those classified as halotolerant, *P. autotrophica* and *P. dioxanivorans* are described as able to grow with CO_2 as the only carbon source, while *P. carboxydivorans* uses carbon monoxide (CO) as a sole source of carbon and energy (Park et al., 2008) and potentially CO_2 as carbon source (Grostern and Alvarez-Cohen, 2013). In this study, chemolithoautotrophic growth (CO_2/H_2) was detected in *P. autotrophica* and *P. dioxanivorans*. On the other hand, *P. carboxydivorans* did not grow with these substrates. *Pseudonocardia* species accumulated mainly hydroxyectoine when growing with 3 % NaCl. Ectoine was not detected during the growth of *P. dioxanivorans*; this species accumulated the highest amounts of hydroxyectoine (4 % of the dry biomass) of all, at the salinity range tested. However, the doubling times obtained for *Pseudonocardia* species in liquid phase were very long. *Pseudonocardia* spp. display a typical Actinomycete filamentous morphology with very slow growth (Franco and Labeda, 2014). This genus has received attention due to the wide range of valuable chemicals that it can produce, such as antibiotics and immune-modulating factors (Riahi et al., 2022), however, the slow growth of *Pseudonocardia* spp. has been identified as a drawback for its implementation in cell factories. Thus, the *Pseudonocardia* species (Seipke et al., 2012) identified in this study were not selected for their further optimization in TS2.

Table 1
Average values (n = 5) of the parameters measured in TS1 for each of the strains growing at 3 % NaCl.

Strain	Doubling Time (hours)	CO_2 removal ($g_{CO2(g)}$ $g^{-1}_{(l)}$)	Ectoine content (mg_{Ect} g^{-1})	Hydroxyectoine content (mg_{HEct} g^{-1})
Alkalilimnicola ehrlichii	–	N.D.	N.D.	N.D.
Hydrogenibacillus schlegelii	25.7 ± 1.0	1.1 ± 0.2	4.1 ± 1.9	18.6 ± 5.6
Hydrogenovibrio marinus	7.9 ± 0.9	2.3 ± 0.2	26.7 ± 3.1	N.D.
Pseudonocardia autotrophica	242.0[a]	0.6 ± 0.1	7.3 ± 5.8	13.4 ± 0.3
Pseudonocardia carboxydivorans	–	N.D	N.D	N.D
Pseudonocardia dioxanivorans	81.3[a]	0.7 ± 0.1	N.D	41.4 ± 9.1
Rhodococcus opacus	15.9 ± 3.6	1.1 ± 0.4	17.8 ± 1.3	5.0 ± 2.1

[a] The biomass of the replicates was measured filtering the total volume of a bottle at each time point. The value was not calculated as average. N.D. Not detected.

Hydrogenibacillus schlegelii, Hydrogenovibrio marinus, and *Rhodococcus opacus* were able to use CO_2 with H_2 with doubling times inferior to 24 h (Table 1). All these species produced ectoine chemolithoautotrophically with 3 % NaCl (Table 1); highest product yield of ectoine was obtained for *H. marinus* (3 % of the dry biomass). *R. opacus* and *H. schlegelii* accumulated hydroxyectoine in addition to ectoine (25 % and 82 % of hydroxyectoine out of the total ectoines pool, respectively). Both, *R. opacus* and *H. schlegelii* have the *ectD* gene and hydroxyectoine production was expected. However, the high amounts of hydroxyectoine natural production detected at 3 % of NaCl in *H. schlegelii* are probably related to its thermophilic nature. Hydroxyectoine, in contrast to ectoine, is a good glass-forming compound as a result of the stronger intermolecular H-bonds with the OH group. It displays remarkable desiccation protection properties which explains its accumulation in response to elevated temperature (Tanne et al., 2014; Van-Thuoc et al., 2013). Moreover, hydroxyectoine production is more prominent in minimal medium, rather than in rich medium (Tao et al., 2016). Thus, according to these results, the use of a thermophilic and autotrophic bacteria can be a good approach to produce hydroxyectoine as the sole osmoprotectant without the use of genetically modified bacteria.

The strains *H. schlegelii*, *P. dioxanivorans* and *P. carboxydivorans*, according to literature, are able to grow with CO as the carbon source

Table 2
Ectoine production by selected strains with varying H_2/O_2 ratios.

H_2/O_2 headspace (%)	H_2 removed (%)	Biomass (mg L^{-1})	Ectoine content (mg_{Ect} g^{-1})	Hydroxyectoine content (mg_{Hect} g^{-1})
Hydrogenibacillus schlegelii				
70/6.3	*9.2*	32.6 ± 2.1	*2.6 ± 0.2*	*5.7 ± 0.7*
60/8.4	15.2	64.7 ± *11.7*	1.9 ± 4.6	*15.3 ± 2.3*
50/10.5	*19.6*	84.8 ± *5.9*	*2.5 ± 6.7*	*12.7 ± 2.9*
40/12.6	*28.1*	96.9 ± *12.3*	*2.1 ± 1.9*	*19.6 ± 8.9*
30/14.7	*30.7*	106.5 ± *8.9*	*0.6 ± 0.3*	*24.8 ± 7.6*
Hydrogenovibrio marinus				
70/6.3	*11.1*	26.6 ± 5.3	*33.1 ± 5.5*	*ND*
60/8.4	12.2	44.7 ± *8.2*	27.0 ± 5.0	
50/10.5	*15.7*	63.0 ± *0.4*	*32.4 ± 4.2*	
40/12.6	*24.7*	89.3 ± *2.3*	*26.2 ± 2.8*	
30/14.7	*30.3*	96.1 ± *0.5*	*31.9 ± 5.3*	
Rhodococcus opacus				
70/6.3	*12.8*	56.6 ± 8.3	*19.3 ± 3.3*	*6.1 ± 1.3*
60/8.4	16.7	82.9 ± *24.7*	17.9 ± 4.4	6.1 ± 1.5
50/10.5	*24.1*	87.1 ± *6.7*	*16.6 ± 3.4*	*3.6 ± 3.6*
40/12.6	*30.3*	113.0 ± *21.1*	*16.7 ± 0.07*	*5.7 ± 1.1*
30/14.7	*29.8*	188.9 ± *20.6*	*17.3 ± 3.4*	*5.5 ± 0.4*

N.D. Not detected.

(Grostern and Alvarez-Cohen, 2013; Krüger and Meyer, 1984; Park et al., 2008). Thus, the autotrophic transformation of CO into ectoines was also tested. Only *H. schlegelii* produced hydroxyectoine using CO as the only substrate (1.4 %), while in *P. dioxanivorans* the production of hydroxyectoine was only detected if H_2 and CO_2 were present (see supplementary material). This could be related to the slow growth of Actinomicetes or to their carboxydovoric nature (Grostern and Alvarez-Cohen, 2013).

3.3. Best environmental conditions to enhance CO_2 transformation into ectoines

Overall, the best performance species for the development of a production platform for ectoines from CO_2 were *H. schlegelii*, *H. marinus,* and *R. opacus* due to their fast growth and their high ectoines accumulation. Thus, these were the selected species to conduct TS2.

3.3.1. Effect of oxygen-hydrogen ratios

An interesting approximation is the fixation of CO_2 with H_2 at low oxygen concentrations. *H. schegelii* is considered an anaerobic facultative bacterium (Maker et al., 2017), while *R. opacus* is able to reduce nitrate (Alvarez et al., 2019). Both of them fix CO_2 using the RubisCO enzyme, although, they also possess the key genes for CO_2 fixation through the reverse TCA cycle (typical of anaerobes) (Maker et al., 2017). Hence, in this assay the bacteria was exposed to different ratios of oxygen and H_2, when excess of nitrate was present, to study the potential production of ectoines in the absence of oxygen. The results obtained are shown in Table 2.

According to the stoichiometric Eq.4 (4) (Yu and Lu, 2019) to oxidize 100 % of hydrogen, 50 % of oxygen is required.

$$O_2 + 0.5\ H_2 \rightarrow 0.5\ H_2O\ E^{0\prime}\ (V) = 0.81\ \Delta G^{0\prime}\ (kJ/mol) = -78.1 \qquad 0.25\ (4)$$

In all the scenarios where there was stoichiometric oxygen limitation, hydrogen was not oxidized and growth stopped. Thus, growth and the production of ectoines in the absence of oxygen was discarded.

The content of ectoine was not affected by the absence of oxygen in the case of *H. marinus* and *R. opacus*. Although, higher ectoine productivities were obtained in assays with more than 15 % O_2 in the headspace. In the case of *H. schlegelii*, lower contents of hydroxyectoine were detected when oxygen was not present. Although *H. schlegelii* is a facultative anaerobe, its growth at high salinity and temperature requires the production of hydroxyectoine. Ectoine can be produced in the absence of oxygen, however, the EctD protein hydroxylates ectoine in a reaction dependent on iron(II), molecular oxygen, and 2-oxoglutarate (Bursy et al., 2007). Thus, even if *H. schlegelii* was able to oxidize H_2 anaerobically, it could not survive at high salinity and temperature in the absence of oxygen since hydroxyectoine would not be produced.

According to these results, further tests were done with an atmosphere of 30 % of H_2 and excess of O_2.

3.3.2. Effect of salinity

Salinity is a requirement to promote the expression of *ect* genes (Argandoña et al., 2021). None of the strains tested could grow at salinities above 8 % NaCl. *R. opacus* was the only bacteria able to grow at 7 %, while *H. marinus* grew up to salinities of 6 %, and *H. schlegelii* grew at maximum salinities of 5 % using CO_2 as the carbon source and H_2 as the energy source (Fig. 3). *H. marinus* accumulated the highest amount of ectoine among the three tested strains. With 3 % NaCl, ectoine production was 32.8 ± 1.8 mg ectoine g biomass^{-1}, but this yield was doubled when the bacterium was grown with 6 % NaCl (i.e. 79.6 ± 10.5 mg ectoine g biomass^{-1}). Hydroxyectoine was not detected, which was

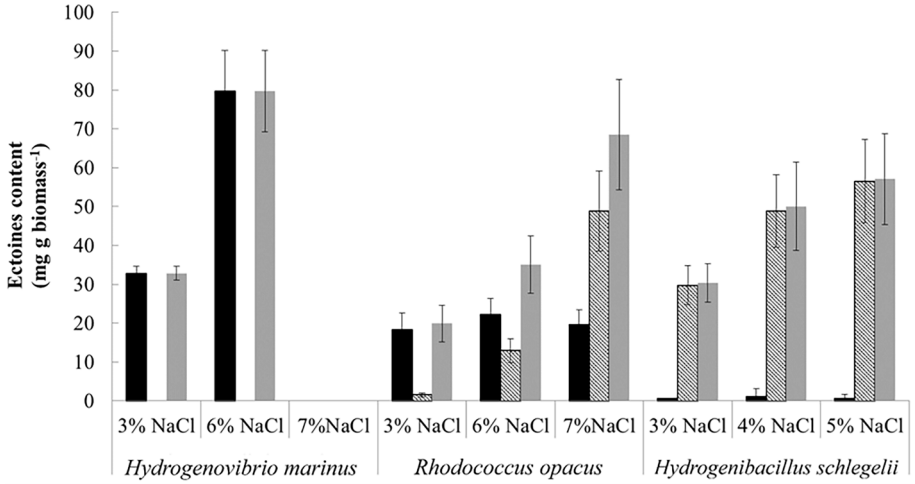

Fig. 3. Average intra-cellular ectoine and hydroxyectoine content at different salinities (% NaCl). Black column, ectoine; dashed column, hydroxyectoine, and grey column, total ectoines.

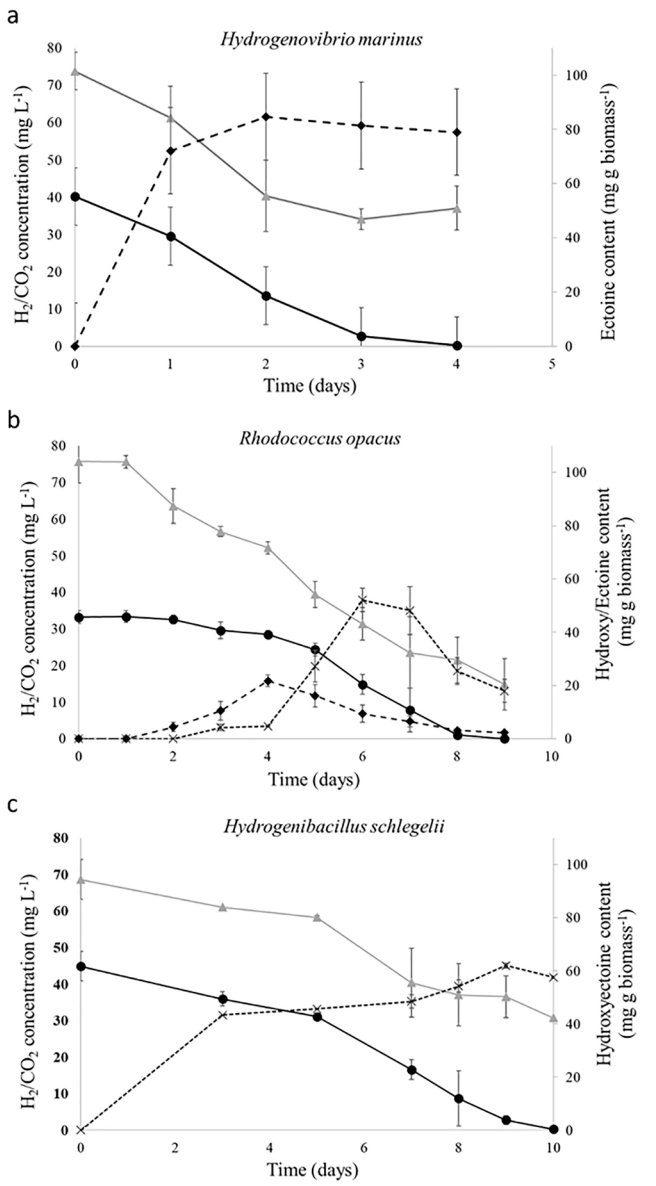

Fig. 4. Time course of the concentration of H_2 (●, black line), CO_2 (▲, grey line) and content of ectoine (◆, dashed line) and hydroxyectoine (*, slashed line) during a) *H. marinus* cultivation at 6% NaCl, b) *R. opacus* cultivation at 7% NaCl and c) *H. schlegelii* cultivation at 5% NaCl.

expected since *H. marinus* lacks the *ectD* gene. *R. opacus* produced 18.3 ± 4.2 mg ectoine g biomass^{-1} at 3 % NaCl. As it has been observed before for other halophiles, the relative abundance of hydroxyectoine increased at higher salinities (Tao et al., 2016) in *R. opacus*, while keeping similar contents of ectoine. When the salinity was risen to 6 % and 7 % NaCl, this bacterium increased the production and accumulation of hydroxyectoine to values of 12.9 ± 3.1 and 48.9 ± 10.3 mg hydroxyectoine g biomass^{-1}. In the case of *H. schlegelii* the main osmoprotectant detected was hydroxyectoine, whose content reached values of 48.8 ± 7.3 and 56.5 ± 10.7 mg hydroxyectoine g biomass^{-1} at 4 and 5 % NaCl. The higher hydroxyectoine content observed at high salinities is related to its superior protective effect and important protective function against desiccation in comparison to its precursor ectoine (Tao et al., 2016).

3.4. Ectoine and hydroxyectoine linked to CO_2 depletion over time

A final study was carried out combining the optimum parameters from previous tests. CO_2 removals of 3.1, 3.4 and 3.5 $g_{CO2}\ g^{-1}_{biomass}$ were obtained for *H. schlegelii, H. marinus* and *R.opacus.* The pH increased in all the cases from 0.16 to 0.2 units during CO_2 removal.

Fig. 4 shows ectoine and hydroxyectoine production along time on the batch bioreactors. *H. marinus*, although only accumulated ectoine, had the fastest growth at 6 % NaCl with the shortest lag phase. Ectoine production was detected on the first day with values of 72.2 ± 10.7 mg of ectoine g biomass^{-1} and reached maximum values of ~ 85 in the quadruplicates. When H_2 was depleted ectoine values decreased due to metabolic shift towards the use of ectoine as energy source (Czech et al. 2018). *R. opacus* had a longer lag phase when growing at 7 % NaCl. Ectoine was detected for the first time on day 2 and reached its maximum contents (21.2 ± 2.2 mg of ectoine g biomass^{-1}) on day 4, while hydroxyectoine production was delayed in the early exponential phase and increased at the expense of ectoine when the cells were in late exponential growth (52.1 ± 4.5 and 48.1 ± 9.0 mg of hydroxyectoine g biomass^{-1} by day 6 and 7).

H. schlegelii was able to grow at a maximum of 5 % NaCl. Ectoine content was negligible, while hydroxyectoine was the main osmoprotectant detected. This osmolite was firstly detected by day 3 once the biomass reached contents of 30.7 ± 6.1 mg L^{-1} and increased during exponential growth when H_2 was present to maximum contents of 62.0 ± 7.9 mg of hydroxyectoine g biomass^{-1}. Depending on the bacterial species, hydroxyectoine accumulation exhibits a remarkable growth phase dependence, and its production is at earlier or later stages contingent upon its physiological role (Tao et al., 2016). At high temperature and salinity, hydroxyectoine was more necessary to maintain the cellular integrity and that is probably why it was the only osmolite detected. In fact, a side study was developed to understand the role of temperature on hydroxyectoine production by *H. schlegelii*. Ectoine was detected when the temperature was decreased to 55 °C, while at 65 and 75 °C hydroxyectoine was the only osmolite detected (see supplementary material).

At present, industrial production of ectoines is primarily performed with sugars or rich carbon sources in the presence of very high salinities (15–20 % NaCl) which reduces the product revenue. New research is focusing on the development of ectoine cell factories without salt, however, genetically modified organisms (GMOs) are required. Although, this aspect reduces cost, the main problems encountered are: The low productivities and low stability of non-halophilic GMOs as ectoine producers, and the lack of attraction that GMOs have in the cosmetic, pharmaceutical and medical market. In the case of hydroxyectoine, its single production is a rare event. The majority of bacteria, with the exception of some *Marinococcus* strains and *Pseudomonas stuzeri* (Schiraldi et al., 2006; Seip et al., 2011), produce it as a mixture demanding even more elaborate downstream processes if hydroxyectoine wants to be recovered.

This research demonstrates for the first time the feasibility of producing ectoine with CO_2 and H_2 using relatively low salt concentrations. Additionally, hydroxyectoine was produced as main osmolite by *H. schlegelii* using high temperatures and a minimum medium. In fact, the values of hydroxyectoine obtained by *H. schlegelii* are in the range of the hydroxyectoine yields obtained by non-GMOs consuming glucose (70–130 mg hydroxyectoine g biomass^{-1}). However, this technology still faces some limitations. The ectoine contents achieved in this study, although similar to those obtained with aerobic methanotrophic bacteria (75 mg g biomass^{-1}) (Cantera et al., 2017), were lower than those obtained from sugars (360–540 mg g biomass^{-1}) (Becker and Wittmann, 2020; Liu et al., 2021). This is likely related to the energetic constrains of chemolithoautotrophy. Nevertheless, utilization of CO_2 as carbon source may bring economic and sustainability-related advantages to the process. On the other hand, technological challenges, such as gas–liquid mass transfer limitations, may arise when scaling-up the technology that

can affect maximum ectoine productivities. In this sense, engineering reactors with improved gas–liquid mass transfer may be necessary for the further implementation of this technology. Another consideration is that high salinity promotes corrosion of reactor materials, conducting to extra maintenance costs. Strains identified in this work produce ectoine at salinities 50 % lower (6 % NaCl) than the currently used in the industrial ectoine production from sugars, which can offer advantages.

4. Conclusions

Here a proof-of-concept for the microbial production of ectoines from CO_2 was shown, with the identification of several bacterial strains able to conduct this conversion. Environmental parameters (e.g. salinity) affecting CO_2-to-ectoines conversion were tested and give directions for further optimization. To maximize production, utilization of reactors with improved gas–liquid mass transfer may be key and remain to be tested. This research will give impetus to future implementation of a new biotransformation platform capable of creating value out of CO_2 mitigation using extremophilic autotrophs.

CRediT authorship contribution statement

Sara Cantera: Conceptualization, Investigation, Validation, Visualization, Writing – original draft, Funding acquisition, Supervision. **Francesca Di Benedetto:** Formal analysis, Methodology, Investigation, Visualization. **Ben F. Tumulero:** Formal analysis, Methodology, Investigation. **Diana Z. Sousa:** Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing.

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

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