Biotic and abiotic effects on CO$_2$ sequestration during microbially-induced calcium carbonate precipitation

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One sentence summary: Microbially-induced calcium carbonate precipitation can promote CO$_2$ sequestration through biotic and abiotic mechanisms.

ABSTRACT

In this study, CO$_2$ sequestration was investigated through the microbially-induced calcium carbonate precipitation (MICP) process with isolates obtained from a cave called ‘Cave Without A Name’ (Boerne, TX, USA) and the Pamukkale travertines (Denizli, Turkey). The majority of the bacterial isolates obtained from these habitats belonged to the genera *Sporosarcina*, *Brevundimonas*, *Sphingobacterium* and *Acinetobacter*. The isolates were investigated for their capability to precipitate calcium carbonate and sequester CO$_2$. Biotic and abiotic effects of CO$_2$ sequestration during MICP were also investigated. In the biotic effect, we observed that the rate and concentration of CO$_2$ sequestered was dependent on the species or strains. The main abiotic factors affecting CO$_2$ sequestration during MICP were the pH and medium components. The increase in pH led to enhanced CO$_2$ sequestration by the growth medium. The growth medium components, on the other hand, were shown to affect both the urease activity and CO$_2$ sequestration. Through the Plackett–Burman experimental design, the most important growth medium component involved in CO$_2$ sequestration was determined to be urea. The optimized medium composition by the Plackett–Burman design for each isolate led to a statistically significant increase, of up to 148.9%, in CO$_2$ uptake through calcification mechanisms.

Keywords: ureolysis; calcium carbonate; cave; travertine; CO$_2$ sequestration; calcification

INTRODUCTION

Carbon sequestration consists in transferring and storing atmospheric carbon dioxide (CO$_2$) into other forms of carbon or by injecting into non-atmospheric reservoirs (e.g. depleted oil and gas reservoirs, non-mineable coal seams, deep saline formations and Deep Ocean). The conversion of CO$_2$ into other forms of carbon is a process that can occur through natural and anthropogenic processes (Lal 2008). For many years, plants and photosynthetic microorganisms were considered to be the only natural sinks for CO$_2$.

More recently, researchers have identified some microorganisms capable of sequestering CO$_2$ through carbonate precipitation process, which has been called biomineralization or ‘microbially-induced carbonate precipitation (MICP)’ (Okwadha and Li 2010; Gray and Engel 2013). Researchers suggest that MICP occurs as a by-product of urea hydrolysis with urease enzymes produced by ureolytic bacteria (Douglas and Beveridge 1998; Castanier, Le Métayer-Levrel and Perthuisot 1999; De Muynck, De Belle and Verstraete 2010; DeJong et al. 2010; Salek et al. 2013). The suggested chemical reactions for MICP through ureolysis for CO$_2$ sequestration are presented below (Mitchell et al. 2010):

Ureolysis:

\[
CO(NH)_2 + 2H_2O \rightarrow 2NH_4^+ + CO_3^{2-}
\]
CO₂ dissolution in aquatic systems:

\[ \text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \]  \hspace{1cm} (2)

\[ \text{H}_2\text{CO}_3 \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \]  \hspace{1cm} (3)

\[ \text{HCO}_3^- \rightleftharpoons \text{CO}_3^{2-} + \text{H}^+ \]  \hspace{1cm} (4)

Overall ureolysis and CaCO₃ precipitation:

\[ \text{NH}_2\text{CONH}_2 + 2\text{H}_2\text{O} + \text{Ca}^{2+} \rightarrow 2\text{NH}_4^+ + \text{CaCO}_3 \]  \hspace{1cm} (5)

In the ureolysis reaction, urea is degraded by ureolytic microorganisms, resulting in the production of ammonium (NH₄⁺), dissolved inorganic carbon and increasing in pH (Equation 1), which will favor CaCO₃ precipitation in the presence of calcium ions (Equation 5). At alkaline pH values generated by the ammonium production during ureolysis, the CO₂ from the air will dissolve in water and get converted to carbonates (Equations 2-4). These carbonates can, then, react with calcium ions and precipitate as calcium carbonates.

In the MICP process, there are two possible CO₂ sources for the calcification reaction to happen: (i) CO₂ from the air and (ii) CO₂ from the bacterial ureolysis and respiration. However, little is known about how much of the CO₂ can really be sequestered by these ureolytic microorganisms in aquatic environments and what are the real roles of microorganisms in the CO₂ sequestration during MICP. Studies investigating MICP have been done mostly with microorganisms from soil. In soils, several species have been described to be able to perform MICP, such as *Sporosarcina pasteurii* (Meyer et al. 2011), *Pseudomonas calcis* (Boquet, Boronate and Ramos-Cormenzana 1973), *Bacillus* sp. VS1 (Chu, Stabnikov and Ivanov 2012) and *P. denitrificans* (Hamdan 2011). However, the presence of these microorganisms in pristine aquatic environments, such as caves and travertines, as well as their roles in calcification and CO₂ sequestration has not been fully investigated.

Recent studies suggested that the formation of stalactites and stalagmites in natural karst landscapes, such as caves and travertines, could involve microorganisms active in MICP (Banks et al. 2010). In such habitats, it has been hypothesized that the presence of CO₂, high calcium concentrations and the presence of urea provide optimal environmental conditions for biomineralization and therefore formation of stalactites and stalagmites and CO₂ sequestration (Liu et al. 2010). The fact that these formations have been in these karstic environments for several thousands of years and continue to grow every year, suggest that, if MICP is truly involved in the production of these formations, it could be a very promising natural long-term carbon sequestration process.

In these karstic environments, calcium comes originally from the rocks and surrounding soils (Kumaresan et al. 2014). Urea, on the other hand, is brought into caves and travertines through different sources: in the case of caves, urea is frequently introduced through mammals’ urine (Johnston, Muench and Banks 2012), such as bats, and from seasonal or continuous water infiltration from the surface (Jameson and Alexander 1994; Baker and Fairchild 2012; Ortiz et al. 2013). Compared to caves, travertines are outdoors, more prone to urea contamination from diverse sources, such as recreational human activities, animal excretions, municipal or agricultural wastewater infiltration (Amundson and Kelly 1987; Kacaroglu 1999). The fact that urea and calcium are present in these habitats allows us to hypothesize that microorganisms involved in MICP could also be present in these aquatic environments and be playing an important role in calcium carbonate precipitation and carbon sequestration.

Vis-a-vis the importance of ureolytic microorganisms in the MICP process and the fact that 10% of the Earth’s surface is occupied by karst landscapes, the presence of ureolytic microorganisms was investigated in two natural karstic environments, the Pamukkale travertines in Denizli, Turkey and the ‘Cave Without A Name’ in Texas, USA. In this study, we isolated and identified ureolytic microorganisms and determined their role in MICP and CO₂ sequestration. The effects of growth conditions on the carbon sequestration by these microorganisms were also investigated to better understand their physiology and roles in carbon sequestration during MICP.

**MATERIALS AND METHODS**

**Sample collection and characterization**

Water samples were collected aseptically in sterile 1L polyethylene containers from the Pamukkale travertine ponds in Denizli, Turkey (37° 54′ 59″ N, 29° 07′ 02″ E), and a pond in the ‘Cave Without A Name’ in Boerne, TX, USA (29° 47′ 40″ N, 98° 43′ 55″ W) in December 2011 and March 2012, respectively. The samples were kept at 4 °C until they were processed. The physicochemical characteristics of the water samples were measured in triplicate according to the standard methods of water analysis (APHA 2005) and presented in Table S1 (Supporting Information). Water samples were stored at 4 °C until brought to the laboratory for further analyses.

**Growth conditions for enrichment and isolation of calcifying bacteria**

Well-mixed aliquots of the water samples were inoculated (10%, v/v) in 13 different growth media as described in Table S2 (Supporting Information), and incubated aerobically at 150 rpm (INNOVA 44, New Brunswick Scientific Co., USA) for 3 d at 28 and 20 °C for travertine and cave samples, respectively. These temperatures were based on the local temperatures during sampling. For the bacterial isolation, serial dilutions were done in phosphate buffer solution (PBS, 0.01M, pH = 7.4), and samples were plated in triplicates onto agar plates prepared with 15 g L⁻¹ of agar with their respective original growth broth (Table S2, Supporting Information). The plates were incubated for 24 h at 28 and 20 °C for the travertine and cave samples, respectively. Different colonies in each plate were selected based on colony morphology. The selected colonies were streaked at least three times under the same conditions to ensure purity.

The isolates were then investigated for urease activity using the Stuart's broth and the Christensen's agar plates. The Stuart’s broth contained 0.1 g L⁻¹ yeast extract, 9.1 g L⁻¹ KH₂PO₄, 9.5 g L⁻¹ K₂HPO₄, 20 g L⁻¹ urea and 0.01 g L⁻¹ phenol red (Stuart, Stratum and Rustigian 1945). The Christensen's agar plates contained 1 g L⁻¹ peptone, 1 g L⁻¹ dextrose, 5 g L⁻¹ NaCl, 2 g L⁻¹ KH₂PO₄, 20 g L⁻¹ urea, 0.012 g L⁻¹ phenol red and 16 g L⁻¹ agar (Christensen 1946). All the urease-positive isolates were also investigated for calcium carbonate precipitation, according to Hammes, Boon and de Villiers (2003). All the calcifying isolates were stored in 50% glycerol (v/v) and kept at −80 °C as stock cultures for future experiments. In all experiments, unless indicated otherwise, the travertine and cave isolates were grown in their specific isolation medium, as described in Table S2 (Supporting Information), under 150 rpm at 28 and 20 °C, respectively.
DNA extraction and amplified ribosomal DNA restriction analysis (ARDRA)

The total DNA of each isolate was extracted with the DNeasy® Blood and Tissue Kit as described by the manufacturer (Qiagen 2006). DNA quantification (O.D. 260) and quality (ratio A260/A280) were determined with the Take3 plate of the Synergy MX Microplate reader (BioTek, USA). Total DNA extracted from each isolate was stored at −20 °C until further analyses.

The ARDRA experiment was performed with the universal eubacterial primers for the 16S rRNA genes. The primers were 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1401r (5′-CGGCTGCTACAGGACC-3′) (Rodrigues et al. 2009). Triplicate PCR reactions of 50 μL for each isolate were performed using 1X AmpliTaq Gold® Fast PCR Master Mix (Applied Biosystems), 0.2 μM of each primer and 50 ng μL⁻¹ of the DNA template in the Applied Biosystems Veriti™ thermal cycler. The PCR amplification conditions were performed as previously described (Rodrigues et al. 2009). All replicate experiments contained negative controls without DNA and positive controls containing DNA from Escherichia coli.

In order to verify the PCR amplification, aliquots of 10 μL of the PCR products were mixed with 5 μL of loading dye (Thermo Scientific), and loaded onto 1% agarose gel (Omnipur Agarose, EMD Millipore) containing SYBR Safe DNA gel stain (Invitrogen) (5 μL SYBR Safe stain per 50 mL TAE buffer), using 1X TAE buffer (Fermentas) as the running buffer. The agarose gel electrophoresis was run at 150 V for 30 min. The DNA in the gel was visualized and photographed with a Compact Digidig System UVDI, Major Science. The DNA fragment sizes were estimated using a 100 bp DNA ladder (Thermo Scientific). The remaining 40 μL of amplified PCR products were purified for further analyses using the QiAquick PCR Purification Kit (Qiagen), as described by the manufacturer (Qiagen 2008).

The triplicate products for each isolate were combined in a single tube for purification. The purified products were quantified and their concentrations were adjusted to 100 ng μL⁻¹ using the Take3 plate.

The 16S rRNA gene amplicons were then digested with Hhal (GGG/C) and MspI (C/CGG) (New England Biolabs). Briefly, for each isolate, 10 μL of the purified PCR products was digested individually with each restriction enzyme in a reaction mixture containing 1 μL of restriction enzyme (20 μM), 2 μL of Buffer-4 (New England Biolabs), 6.8 μL of nuclease free water (Thermo Scientific) and 0.2 μL of bovine serum albumin (Thermo Scientific). All digestions were incubated at 37 °C for 3 h, followed by a denaturation step at 65 °C for 20 min. The restriction fragments were separated by electrophoresis in 3% (w/v) agarose gel at 60 V for 4 h and visualized as previously described (Pourshafie, Vahdani and Popoff 2005). ARDRA patterns were analyzed using the PyElph1.3 software that automatically extracts the data from the gel images, computes the molecular weights and compares the DNA patterns using the neighbor-joining method to determine the percent similarity among the isolates (Pavel and Vasile 2012).

16S rRNA phylogenetic analyses

The purified PCR fragments were sequenced by the MD Anderson Genetic Laboratories (Houston, TX, USA). Both the forward and reverse primers were used to sequence the whole length of the PCR products. The sequences were assembled using MEGA 5.1 software. The sequences obtained were compared to similar sequences available at the National Center for Biotechnology Information (NCBI) using BLASTn (Altschul et al. 1990). Similar sequences to the isolates were retrieved from GenBank and aligned against the DNA sequences of the isolates using MEGA 5.1 (Tamura et al. 2011). A phylogenetic tree was also constructed using MEGA 5.1 through the neighbor-joining method (Saitou and Nei 1987). Bootstrap analysis based on 500 replications of the neighbor-joining dataset was also obtained. The nucleotide sequences determined in this study were deposited in the NCBI database under accession numbers KM009125 to KM009137.

Determination of physiological properties of calcifying isolates

Physiological properties of the isolates were determined by measuring the growth rate of each isolate at different pH values and temperatures in their specific isolation medium. A plate reader was used to obtain the microbial growth rates in each experiment by measuring the absorbance at 600 nm for 24 h in each well. To determine the pH range that isolates were able to grow, 96-well plates were prepared for each isolate containing 300 μL of its specific growth medium in triplicate with pH values of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. For the pH experiments, the incubation temperature was kept at 28 and 20 °C for the travertine and cave isolates, respectively. For the temperature experiments, each isolate was grown in its specific isolation media at 15, 20, 25, 30, 35 and 40 °C. The pH was kept at 7 ± 0.3 for the temperature experiments. All conditions were performed in triplicate. The growth rates for each condition and isolate were calculated as previously described (Onal Okyay and Frigi Rodrigues 2014). The growth rate results were used to determine the optimum pH and temperature for each isolate. The triplicate results were averaged and the standard deviations were also calculated.

Determination of urease activity, calcification and CO₂ sequestration

Urease activities of calcifying isolates were determined as previously described (Onal Okyay and Frigi Rodrigues 2013). Briefly, color change in the Stuart’s broth was measured at 560 nm every 30 min for 24 h using the 96-well plate reader. At the end of the incubation, the color change rates (h⁻¹) were calculated for each isolate. A standard curve with the purified Jack Bean urease enzyme was used to determine the enzymatic activity (U mL⁻¹) of each isolate. One unit of urease activity corresponds to the amount of enzyme that hydrolyzes 1 μM of urea per min.

The rates of calcification and CO₂ sequestration were determined with five environmental isolates. Among these five isolates, three of them presented the highest urease activities (TR1, TR3 and TR20), while the other two showed the lowest urease activities (TR12 and CV1). TR and CV abbreviations correspond to travertine and cave isolates, respectively. For the calcification assay, these five isolates were incubated in their respective isolation medium supplemented with 6.9 g L⁻¹ calcium chloride, which was found to be the optimum calcium concentration for calcification in S. pasteurii ATCC 11859 (Onal Okyay and Frigi Rodrigues 2014). The remaining calcium ions in the medium, after calcification, were measured by atomic absorption spectroscopy (Perkin Elmer AAnalyst™ 200) after centrifuging at 10 000 rpm for 10 min and filtering the supernatant through 0.2 μm syringe filters. Negative controls, which had no bacterial, were used to determine whether there were non-biological calcite precipitations throughout the experiment.
In order to confirm the CO₂ sequestration, headspace CO₂ concentrations were measured in sealed serum bottles using a gas analyzer containing a non-dispersive infrared CO₂ sensor (Columbus Instruments Model 180C Gas Analyzer, Columbus, OH, USA). For this experiment, overnight grown cultures were centrifuged at 10 000 rpm for 10 min, and cells were collected, washed with sterile PBS and the absorbance of the cell suspensions was adjusted to 0.5 at 600 nm. Subsequently, 39 mL serum bottles containing 9 mL of growth medium (with or without 6.9 g L⁻¹ CaCl₂) were inoculated with 10% of the washed cell suspensions. The bottles were capped with butyl rubber stoppers and aluminum seals. Pure CO₂ gas was injected into each serum bottle to obtain 10% of CO₂ gas in the headspace. The serum bottles were then incubated in the dark for 24 h at 150 rpm and 20 or 28 °C, depending on the isolate origin. After 1, 3.5, 6.5, 9, 21.5 and 24 h, duplicate serum bottles were analyzed for CO₂ concentration in the headspace. All experiments were repeated three times in three different days to take into consideration daily variations. Abiotic controls, which had no cells, were included in each experiment as negative controls. All results were averaged out and their respective standard deviations were also calculated.

Abiotic effects of pH on CO₂ sequestration

Since bacteria can change the pH during growth, the abiotic effect of pH on calcium carbonate precipitation and CO₂ sequestration was also determined in growth medium with adjusted pH and without bacteria (Table S11, Supporting Information). Briefly, similarly to the bacterial CO₂ sequestration experiment, 9 mL of non-inoculated growth medium was placed into 39 mL serum bottles. The pH of the different growth media used in this investigation were adjusted to 7.3, 8.3, 8.9, 9.2 and 9.3. The selection of the pH to do the abiotic experiment in each growth medium depended on the final pH after growing the bacteria in their specific growth medium (see Table 2). After adjusting the pH, all the bottles were capped, sealed, injected with CO₂ and incubated as described in the previous section. After 24 h incubation, CO₂ concentrations in the headspace and the final pH of the solutions were measured.

In order to determine whether the CO₂ in the air was converted to carbonate and/or bicarbonate ions in the different growth media at different pH values, titrations of the media were done according to the standard methods (APHA 2005) (Table S10, Supporting Information). Before starting the incubations, the prepared growth media were titrated with 0.1 N H₂SO₄ with the help of phenolphthalein and methyl orange to determine the initial concentrations of the carbonate ions in the solution. At the end of the incubation periods with or without bacteria, the media were titrated again. These experiments allowed us to distinguish the biotic (i.e. bacteria) and abiotic (i.e. pH) effects on the CO₂ sequestration. All experiments were performed in triplicate in three different days. The results were averaged and the standard deviations were calculated.

Determination of dry cell weight

In order to normalize the CO₂ sequestration results by dry cell biomass, dry cell weights were determined for the five isolates (TR1, TR3, TR20, TR12 and CV1). Each isolate was grown overnight under its specific growth conditions, and a volume of 10 mL from each suspension was filtered through 0.2 μm pore-size sterile filters (Whatman). After filtration, the filter membranes containing the bacteria were dried at 60 °C for 24 h and kept in the desiccator until measured. The dried biomass was determined by calculating the difference in the filter mass before and after filtering the cells in the filter membranes. Experiments for each isolate were conducted in triplicate and the average biomass of dried cells per filter was compared with the control filter membranes after filtration of the media without bacteria. This procedure allowed us to determine any mass change in the filter caused by the media constituents retained in the filter membranes.

Scanning electron microscopy and energy dispersive X-ray spectroscopy

The calcification was confirmed using scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) analyses (JEOL JSM-6010LA). The five isolates (TR1, TR3, TR20, TR12 and CV1) were incubated for 96 h with sterile glass slides in their original isolation medium (Table S2, Supporting Information) with 6.9 g L⁻¹ calcium. At the end of the incubation time, the glass slides were removed with a sterile spatula and washed with sterile distilled water to remove dissolved salts and loosely attached cells. The glass slides were dried at 60 °C overnight and sputter coated (Denton Desk V) with gold at 5 mA for 30 s using 0.00 016 mbar Argon prior to the SEM-EDS analysis. All experiments were done in triplicates and repeated three times in different days.

Growth medium components affecting the CO₂ sequestration

The growth medium components affecting microbial CO₂ sequestration were investigated following the Plackett–Burman (PB) design using the Design Expert software (Khamhbaty et al. 2007). In the design, a total of 11 components commonly found in the medium for MICP microorganisms (tryptone, yeast extract, tricine, ammonium sulfate, glutamic acid, urea, meat extract, peptone, sodium chloride, nickel (II) chloride and glucose) were selected for the CO₂ sequestration investigation. The experimental design is shown in Table S3 (Supporting Information), where each row represents one experiment and each column represents an independent variable.

In all the PB design experiments, 6.9 g L⁻¹ calcium chloride was added to the media. The analysis of variance was used to determine the statistical significance of the results. The quality of the experiments was determined statistically by the coefficient of determination R² and the F-test. These results determined the optimum concentrations of the medium constituents to enhance the CO₂ sequestration for the five isolates (TR1, TR3, TR20, TR12 and CV1). In order to confirm the CO₂ sequestration optimization results, experimental assays were performed under the optimum conditions determined by the Design Expert software and CO₂ sequestration yields were calculated. All experiments were performed in triplicate and the results were averaged out and standard deviations were calculated.

RESULTS

Isolation of calcifying bacteria

In this study, 13 different media (Table S2, Supporting Information), commonly used in MICP studies, were used for the first time to obtain calcifying microorganisms from water samples collected in Pamukkale travertines from Turkey and 'A Cave
Without a Name’ from the United States. The different media yielded different numbers of isolates with distinct morphological characteristics, such as size, shape and color. It is important, however, to point out that some growth media did not allow the growth of any microorganisms from these habitats, this observation was especially true for the cave sample. For instance, the nutrient-rich media did not allow isolation of microorganisms from the cave sample. In total, 6 and 28 isolates from the cave and the travertine pond, respectively, were obtained. After isolation, the microorganisms were investigated for urease activity and calcification, and it was determined that all these isolates were capable of MICP.

Genetic fingerprinting and phylogenetic classification of isolates

The isolates were further investigated for identical band patterns by digestion with HhaI and MspI restriction enzymes of the amplified 16S rRNA genes. The different band patterns were then subjected to cluster analysis. The ARDRA results with the HhaI and MspI enzymes could distinguish 17 (Fig. S1, Supporting Information) and 13 (Fig. 1) different strains, respectively. These results showed that the MspI enzyme was more stringent than HhaI in this study. The isolates presenting 100% identical band patterns were considered to be clonal isolates and were not further investigated. The isolates (four isolates from cave and nine isolates from travertine) presenting distinct band patterns and isolates with a similarity lower than 60% (Fig. 1) were further sequenced for identification.

The sequencing results (Fig. 2) showed that the majority of the isolates belonged to two genera, Sporosarcina and Sphingobacterium. The isolates with high similarity to Sporosarcina were CV1 (92%), TR1 (92%), TR20 (91%) and TR3 (91%), while CV4 (87%), CV3 (88%) and CV2 (83%) had high similarity to Sphingobacterium. The isolates TR11 (99%), TR6 (96%), TR28 (94%), TR9 (94%), TR12 (96%) and TR16 (99%) presented high similarity to Bacillus, Brevundimonas, Chryseobacterium, Alcaligenes, Acinetobacter and Stenotrophomonas, respectively (Fig. 2). Based on these results, we were able to isolate more diverse MICP microorganisms in the travertine samples than in the cave. It is possible, however, that other types of medium, not investigated in this study, could have been more appropriate for the isolation of a more diverse microbial population in the cave.

The comparison of the results obtained in Figs 1 and 2 showed that the ARDRA dendrogram obtained with the MspI enzyme is very similar to the 16S rRNA phylogenetic tree. Therefore, it can be concluded that MspI seems to be a better restriction enzyme to identify and cluster calcifying isolates than HhaI. Additionally, the phylogenetic results (Fig. 2) showed that some isolates, even though they had different band patterns in the ARDRA results (Fig. 1), still presented 99% similarity in their 16S rRNA gene sequences. This was the case for some isolates, such as TR6 and TR11, TR1, TR28, CV4 and TR 20. The 16S rRNA phylogenetic tree also showed that the isolates were clustered in six different classes: Bacilli (CV1, TR1, TR20, TR3, TR11), α-Proteobacteria (TR6), Flavobacteria (TR28), Sphingobacteria (CV4, CV3, CV2), γ-Proteobacteria (TR16, TR12) and β-Proteobacteria (TR9). In this study, the most abundant and diverse number of isolates belonged to Bacilli.

Effect of growth media on bacterial isolation

In the present study, rich and mineral media were investigated. The different types of media used allowed the isolation of diverse microorganisms in the two sites. In the case of the cave, isolates were only obtained in more oligotrophic media, such as the M2 medium (minimal medium) and the 10X diluted-rich media. In the case of the travertine, isolates were obtained from both types of media. The rich media, however, allowed the isolation of five distinct bacteria, while eight distinct bacteria were obtained in the mineral or diluted-rich media. Among the media investigated, ATCC-1832-10X medium was the best to obtain the highest number of isolates from the travertine (Table S2, Supporting Information).

Physiological properties of the calcifying isolates

The physiology of these microorganisms was determined by measuring their pH and temperature growth ranges, as well as their optimum temperature and pH for growth. The results show that the isolates were able to grow in a wide range of pH values and presented optimum pH values between 6 and 9 (Table 1). It is important to point out that the isolates (TR1, TR3 and TR20) presenting the highest urease activities among the isolates (Fig. 3) preferred alkaline conditions for growth. In contrast, the isolates with optimum growth at pH values equal or below 7 presented lower urease activities.

In the case of the temperature, the average annual temperature in our sampling cave is nearly 20°C (Cave 2014). Hence, all the cave isolates were able to grow at this temperature; however, only the isolate CV2 showed an optimum growth at 20°C, the others all had higher optimum growth temperatures. Considering that Texas has very warm temperatures, it is possible that the isolates could have come from an external source other than the cave, which would have explained the preference of these isolates for higher temperatures than the normal cave temperature.

In the Pamukkale travertines, the average annual temperature is 35°C at the exit point, where the spring water comes from the underground (Pamukkale 2014). Our sampling site was, however, far from the spring point. Therefore, the water temperature was around 28°C. The different water temperatures at different points in the travertine would explain the different optimum conditions.
Figure 2. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship among calcifying isolates obtained from the Pamukkale travertines and the ‘Cave Without a Name’ with their closest relatives according to NCBI. Tree was constructed by the maximum likelihood method using MEGA 5.1. Bootstrap values above 40% (based on 500 replications) are shown at each node. Bar at the bottom correspond to the fixed nucleotide substitutions per sequence position.

Table 1. The pH and temperatures at which the isolates are able to grow, and their optimum values.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Isolation growth media</th>
<th>pH range of growth</th>
<th>Optimum pH</th>
<th>Temperature range (°C)</th>
<th>Optimum growth temperature (°C)</th>
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<tbody>
<tr>
<td>TR1</td>
<td>ATCC 1832</td>
<td>3–11</td>
<td>9</td>
<td>15–40</td>
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<tr>
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<td>3–12</td>
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<tr>
<td>TR9</td>
<td>NB</td>
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<td>7</td>
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<tr>
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</table>

temperature preferences of the isolates. In all cases, however, the travertine isolates had optimum growth temperatures in the range of the temperatures found at the site.

Besides investigating the optimum pH and the temperature for the growth of the isolates, we also investigated their urease activities; the 13 isolates showed different urease activities ranging between 8 and 0.2 U mL$^{-1}$ (Fig. 3). The top three performing isolates, TR20, TR1 and TR3, having 8, 3.6 and 3.5 U mL$^{-1}$ urease activities, respectively; and the two worst performing isolates, TR12 and CV1, having 0.22 and 0.18 U mL$^{-1}$ urease activity, respectively, were selected for the calcification investigations and CO$_2$ sequestration. In these investigations, the goal
Figure 3. Urease activities of travertine (TR) and cave (CV) isolates. All isolates were cultured in their specific growth conditions. All experiments were done in triplicate and the error bars correspond to standard deviations.

Figure 4. The calcium concentrations in the growth media during microbial growth.

In the calcification assays, we determined the amount of calcium left in the medium (Fig. 4) and analyzed all the precipitates formed during the microbial growth. The quantifications of the calcium ions left in the bacterial media showed that each isolate had different calcification capabilities (Fig. 4). The isolate presenting the highest calcium carbonate precipitation was TR20, which precipitated almost 100% of the calcium carbonate within 6 h. The isolates TR1 and TR3, on the other hand, precipitated 69 and 87% of calcium carbonate, respectively, in the same period of time (Fig. 4). No changes in the calcium ions in the medium were observed in the control samples, which had no bacteria.

In order to further confirm that the precipitates in the medium were made of calcium, SEM and EDS analyses of the precipitates were performed. The results showed that the particles precipitated by the microorganisms during MICP were CaCO$_3$ mineral precipitates. In Fig. 5A, the SEM results demonstrated that the precipitates are composed mostly by calcite and a few vaterites (Banks et al. 2010). Elemental analysis of the precipitates with EDS (Fig. 5B) confirmed the presence of CaCO$_3$.

**Effects of pH on biotic and abiotic CO$_2$ sequestration**

In aquatic environments, the carbonate system plays an essential role in the abiotic CO$_2$ sequestration (Lai 2008). The carbonate system is also directly linked to pH, which can lead to the formation of carbonate and/or bicarbonate in the water or growth medium (Bustos-Serrano 2010). Microorganisms also play an important role in changing the pH of their surrounding environment and producing CO$_2$ during growth. At the same time, MICP microorganisms have also been described to facilitate calcite precipitation to store CO$_2$ (Okwadha and Li 2010). However, no study so far has done a systematic investigation to determine how much of the CO$_2$ sequestration during the MICP process is really biotic or abiotic.

In this work, the abiotic CO$_2$ uptake was determined by quantifying the CO$_2$ in the headspace of the serum bottles by a gas analyzer and by titration of the media to determine the amounts of carbonate (CO$_3^{2-}$) and bicarbonate (HCO$_3^-$) ions present in the growth media. In this investigation, sterile growth media
Normalized carbon dioxide sequestration rates for each isolate as uptake rates were determined and normalized by the sequestration phenomena happening: abiotic and biotic, by two mechanisms: (1) increasing pH while uptake from the headspace (0.9%). The medium medium ATCC 1832, on the other hand, had the highest abiotic CO₂ uptake (5.4%).

The biotic uptake by the MICP process was also determined by growing the isolates in their respective growth medium and measuring the amount of CO₂ left in the headspace. The results (Table 2) showed that all isolates were able to sequester CO₂. The isolates that showed the highest and the lowest biotic CO₂ uptake were TR3 and TR1 with a capability of sequestering 3.5 and 2.4% of CO₂, respectively (Table 2).

In summary, in the MICP process, we were able to observe two CO₂ sequestration phenomena happening: abiotic and biotic CO₂ uptake. Briefly, most of the isolates increased the pH of the media, which led to an increase in abiotic CO₂ uptake by the growth media, but also the cells induced the calcite precipitation, which also led to CO₂ sequestration. It is worth point out, that if no cells were grown in the media, there would not be any pH increase and therefore no CO₂ removal from the headspace. But, if we changed the pH chemically or biologically to a pH higher than 7, we started to observe CO₂ sequestration when we only had excess amounts of CO₂ (10%) in the serum bottles (see Table S2, Supporting Information).

In addition to the headspace and titration measurements, the CO₂ uptake rates were determined and normalized by the dry cell weight (Fig. 6). Among the isolates, a faster CO₂ sequestration was achieved by the isolate TR20, with a sequestration rate of 90.9 mg mL⁻¹ h⁻¹ (mg dry cell)⁻¹. However, TR1 showed the lowest CO₂ sequestration rate with 12.3 mg mL⁻¹ h⁻¹ (mg dry cell)⁻¹. The comparison of the CO₂ sequestration rates (Fig. 6) with the urease activity results (Fig. 3) showed that the urease activity and the CO₂ sequestration rates were not directly proportional, but were somehow connected. For instance, the isolates having the lowest urease activities and calcification rates (TR12 and CV1) presented significant CO₂ sequestration rates. Based on these results, one could hypothesize that the urease-positive bacteria could be sequestering CO₂ by two mechanisms: (1) increasing pH while growing, which would enhance CO₂ dissolution into the growth medium and induce calcite precipitation as typically observed in the MICP process; and (2) microorganisms could be sequestering CO₂ by unknown CO₂ metabolisms, not necessarily linked to MICP.

Table 2. The changes of CO₂ concentration in headspace and pH of growth media after 24 h incubation with and without bacterial cells to determine biotic and abiotic CO₂ sequestration.

<table>
<thead>
<tr>
<th>Medium (with bacterial cells)</th>
<th>Initial CO₂ in the headspace (%)</th>
<th>Initial pH</th>
<th>Final CO₂ in the headspace (%)</th>
<th>pH after 24 h incubation</th>
<th>CO₂ (%) in abiotic bottles*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR1 in ATCC 1832</td>
<td>10</td>
<td>7.6</td>
<td>2.1</td>
<td>8.9</td>
<td>4.6</td>
</tr>
<tr>
<td>TR3 in NB</td>
<td>10</td>
<td>7.5</td>
<td>2.6</td>
<td>9.2</td>
<td>6.1</td>
</tr>
<tr>
<td>TR20 in NB</td>
<td>10</td>
<td>7.3</td>
<td>3.6</td>
<td>9.3</td>
<td>6.1</td>
</tr>
<tr>
<td>CV1 in NB-10X</td>
<td>10</td>
<td>6.4</td>
<td>8.3</td>
<td>9.1</td>
<td>9.1</td>
</tr>
<tr>
<td>TR12 in ATCC 1832–10X</td>
<td>10</td>
<td>7.3</td>
<td>6</td>
<td>7.3</td>
<td>9.1</td>
</tr>
</tbody>
</table>

*Abiotic bottles had no cells and their pH were adjusted to the same pH as the biotic experiments after 24 h incubation.

Figure 6. Normalized carbon dioxide sequestration rates for each isolate as mg mL⁻¹ h⁻¹ (mg dry cell)⁻¹.

Effects of growth medium components on CO₂ sequestration

The PB experimental design was employed in order to investigate the effects of each growth medium component on the CO₂ sequestration. In the PB design, a total of 11 variables, namely, tryptone, yeast extract, tricine, ammonium sulfate, glutamic acid, urea, meat extract, peptone, sodium chloride, nickel (II) chloride and glucose were investigated. These 11 variables were selected since they are the main components of the isolation media and because media components are known to affect the growth and other cellular metabolisms (Tempest, Meers and Brown 1970). The PB design allowed us to analyze several medium components simultaneously in the growth medium instead of investigating one component at a time. The concentrations of each medium component used in each PB design medium are listed in Table S3 (Supporting Information). After preparing the 16 different PB media, the isolates were grown in each of these media. Calcification and urease activity measurements, followed by CO₂ sequestration determination, were performed for each PB media. The outputs of the PB design, such as each variable’s effects on the urease activity and CO₂ sequestration, p values and the model coefficients, were obtained using the Design Expert software and represented in the supporting information.

According to the PB design results, all of the media contents were found highly significant (p < 0.01) for both CO₂ sequestration and urease activity (Tables S4–S7, Supporting Information). Urea, however, was found to be the most important medium component for both ureolysis (Fig. S3-A, Supporting
Calcite formations in caves and travertines are believed to be formed mainly through the abiotic precipitation of calcium carbonate. Recently, biotic precipitation through MICP has been suggested as a potential mechanism for calcite formations in caves and travertines (Castanier, Le Météayer-Levrel and Perthuisot 1999; Banks et al. 2010). In the MICP, ureolytic microbes have been shown to play an important role in geological formations and to participate in CO$_2$ sequestration (Amundson and Kelly 1987; Fouke, Bonheyo and Sanzenbacher 2003; Fouke 2011). However, there is very little understanding on how environmental bacteria affect the CO$_2$ sequestration through MICP. In the present study, ureolytic environmental isolates were obtained to better understand the relationship between urease activity, MICP, CO$_2$ sequestration and growth conditions. Such understanding will be fundamental for the development of alternative solutions to sequester CO$_2$.

In this study, MICP isolates were obtained, for the first time, from the ‘Cave Without A Name’ and the Pamukkale travertines. The unknown physiology and microbial composition of these habitats led us to investigate 13 different growth media to attempt to obtain diverse microorganisms involved in MICP. The isolation of diverse microorganisms is one of the major challenges for microbiologists, since it requires deep understanding on the physiology and metabolism of microorganisms to simulate conditions as close as possible to their original habitat (Davis, Joseph and Janssen 2005; Stott et al. 2008; Pham and Kim 2012). In order to deal with this problem, different components, including different electron donors, electron acceptors and carbon sources, and various concentrations in media formulations have been used, and researchers have started to isolate more diverse microorganisms (Stott et al. 2008; Pham and Kim 2012). For example, in a previous study, growth medium containing various carbon sources and complex compounds allowed researchers to obtain more diverse isolates than the growth medium having only one carbon source (Alain and Querellou 2009). However, Stott et al. (2008) revealed that no bacterial growth was achieved in defined complex media, such as Nutrient Tryptic Soy or Lucia-Bertani broths, as their samples were geothermal soils. In another study, relatively low concentrations of nutrients present in a diluted growth medium resulted in increasing number of different isolates from environmental samples (Button et al. 1993). Similarly, in our study, we were mostly successful in isolating microorganisms from the cave and travertine using 10x diluted nutrient broth. The preference of our isolates for non-rich growth media could be explained by the oligotrophic nature of caves and travertines, as well as the adaptation of these microorganisms to these habitats (Liu et al. 2010; Ortiz et al. 2014). Therefore, in both of these sites, oligotrophic growth media were more appropriate for the isolations of the microorganisms.

In isolation studies, it is common to obtain many isolates that are actually clonal isolates (Urdaneta et al. 2001; Lagacé et al. 2004). Therefore, researchers use DNA fingerprinting techniques, such as ARDRA, to distinguish these clonal isolates. Previous studies have shown that the sensitivity of ARDRA to identify clonal isolates is dependent on the type of enzyme used (Rodas, Ferrer and Pardo 2003; Wu et al. 2006). Some enzymes, such as Mspl and HhaI, have been described to be more appropriate to distinguish clonal isolates (Pourshafie, Vahdani and Andika 2003; Pereira, Latchford and Mudge 2006). In the literature, it is, however, still a matter of debate which enzyme, Mspl or HhaI, can distinguish better clonal isolates (Pereira, Latchford and Mudge 2006). Hence, we investigated both enzymes with our MICP isolates. In our study, Mspl enzyme was better than HhaI. In fact, the clustering of the phylootypes obtained with the Mspl enzyme was very similar to the results obtained through sequencing, which suggests that Mspl is more suitable for the selection of unique MICP clones.

The sequencing of the unique clones isolated in this study showed the presence of Bacilli, Sphingobacteria, Flavobacteria and
α-, β-, and γ-Proteobacteria in these habitats. Similar observations were also made by previous researchers working with karstic samples (Fouke, Bonheyo and Sanzenbacher 2003; Banks et al. 2010; Fouke 2011). Interestingly, the Sporosarcina genus, which was the predominant genus in our study, was not observed by these researchers in their karstic samples. However, other researchers were able to isolate several Sporosarcina species from soil samples using different isolation media containing urea (Yoon et al. 2001; Hammes, Boon and de Villiers 2003) or not (Claus et al. 1983; Reddy, Matsumoto and Shivaji 2003; Zhang et al. 2010; Bañana 2011).

Based on our findings, it is possible that the addition of urea in all growth media used in this study led to the selection of species having higher urease activities. This finding is also consistent with the conclusions of Hammes, Boon and de Villiers (2003) who observed that urea, when added to the medium for bacterial isolation, led to the selection of ureolytic microorganisms. In fact, we observed that the isolates presenting higher urease activity belonged to the Sporosarcina genus, except for the cave isolate CV1. It is, however, not surprising to see that most of the Sporosarcina isolates presented higher urease activities, since this genus is well known to be ureolytic (Tobler et al. 2011).

Another interesting observation about these isolates with high urease activity was that they had a preference for alkaline pH. This preference for high pH values is typically observed in calcifying microorganisms (Mortensen et al. 2011). This microbial adaptation of ureolytic microorganism to alkaline environments can be explained by the degradation of urea and production of ammonia as a by-product. The production of ammonia leads to pH increase in the surrounding environment. The results obtained are consistent with previous studies that showed that ureolytic bacteria tend to be alkaliphilic microorganisms, with optimum growth at pH values between 8 and 9 (Okwadha and Li 2010). It is important to point out, however, that even though the pH of the original travertine and the cave water samples were 7 and 7.4, respectively, the presence of these microorganisms in sampling sites can be explained by the tolerance of these microbes to wide pH ranges as shown in Table 1. To relate these findings to the environmental samples, in the travertine water sample, we found higher ammonia concentrations than the cave (Table S1, Supporting Information), which would explain the larger number of MICP isolates obtained from the travertine than the cave.

Further investigation of the urease and MICP activities by the isolates determined that microorganisms from the same genus, such as Sporosarcina sp. TR1 and Sporosarcina sp. CV1 or Sphingobacterium sp. CV2 and Sphingobacterium sp. CV4 had different urease and MICP activities. This finding contradicts a previous report that suggested that the urease activity or the rate of MICP was dependent on the genus of the microorganisms (Hammes, Boon and de Villiers 2003). In the calcification and CO₂ uptake experiments during MICP, we observed that calcium carbonate precipitation and the CO₂ uptake by the isolates were completed within 5–10 h, which were mostly in the exponential phases of these microorganisms. The calcite precipitation in the exponential phase has also been previously observed in another study (Castanier, Le Métayer-Level and Perthusiot 1999). In fact, the calcification process has been described to correlate well with the CO₂ uptake as the amount of calcite precipitated was previously described to be directly proportional to the CO₂ sequestrated (Okwadha and Li 2010).

The stoichiometric reaction of ureolysis in the calcium carbonate formation shows that 1 mol of urea hydrolyzed will sequester 1 mol of CO₂ to form 1 mol of calcium carbonate, which precipitates in the medium (Banks et al. 2010; Okwadha and Li 2010), therefore the number of moles of CO₂ sequestered should be equal to the number of moles of calcium carbonate precipitated. This could potentially be true in soils, but is certainly not accurate in aquatic systems, since the CO₂ in the air tend to dissolve in the water or in the growth medium, and depending on the pH of the water, the CO₂ can be converted to carbonates (Weiss 1974; Lee, Kim and Mahanty 2014). Additionally, it is important to point out that bacterial cells release CO₂ due to respiration and can change the pH of the growth medium, which would also affect the carbonate system (Millero 1995). These factors should be taken into consideration in order to clarify the true biotic role of the MICP process in the CO₂ sequestration of aquatic systems.

In order to better understand the biotic and abiotic CO₂ sequestration processes during MICP, we compared the CO₂ concentrations in the headspaces in the growth medium with different pH values (abiotic results), and in the growth medium at the end of the MICP assays (abiotic + biotic results) (Table 2). As suggested by Lee and collaborators, the pH played an important role in the abiotic uptake. For instance, in the abiotic assay (without bacterial cells), the ATCC 1832 medium was able to serve as a CO₂ sink and, hence, decreased the CO₂ content present in the headspace by 5.4%. In the presence of cells (biotic and abiotic process together), however, the CO₂ decreased by 7.8%. These results suggest that the biotic CO₂ uptake was 2.4%. In the case of the NB3 medium, the CO₂ decreased by 3.9 and 3.5% in the abiotic and biotic processes, respectively. In the medium NB, the abiotic and biotic processes were able to sequester 3.9 and 2.5%, respectively. For the isolates TR12 and CV1, their growth media showed almost no effect on the abiotic uptake of CO₂ and the cells were able to sequestered 2.7 and 3.1% of CO₂, respectively. Based on our findings, the five isolates investigated were able to sequester different amounts of CO₂. The results suggested that CO₂ sequestration through MICP is strain-specific and depends on the environmental factors, such as pH.

When we take into consideration the amount of calcium carbonate formed by MICP and the CO₂ dissolved in each medium, we observed that stoichiometrically more CO₂ is being sequestered than we can account for. The conversion of CO₂ sequestered by TR1, TR3, TR20, CV1 and TR12 to moles shows that 0.054, 0.051, 0.043, 0.025 and 0.027 moles of CO₂ is being sequestered, respectively. In addition, when we calculate the number of moles of CO₂ in the CaCO₃ precipitate and carbonates in the growth medium for each isolate, we get $6.4 \times 10^{-4}$, $6.9 \times 10^{-4}$, $6.9 \times 10^{-4}$, $2.6 \times 10^{-4}$ and $1.8 \times 10^{-4}$ moles, respectively. Hence, when comparing these two sequestration results, it is clear that the MICP is not the only CO₂ sequestration mechanism. It is possible that besides the uptake mechanisms observed in this study, there might be other possible CO₂ fixing mechanisms being performed by these microorganisms, such as the Calvin-Benson reductive pentose phosphate cycle, the reductive citric acid (Arnon-Buchanan) cycle, the reductive acetyl-CoA (Wood-Ljungdahl) pathway, the hydroxypropionate (Fuchs-Holo) bi-cycle, the 3-hydroxypropionate/4-hydroxybutyrate and dicarboxylate/4-hydroxybutyrate cycles, as previously described (Ketjinda, Sinchaipanid and Limsuwan 2011). In fact, Ortiz et al. (2014) came across some of the genes involved in these CO₂ fixation pathways when investigating a cave ecosystem.

In this study, for the different isolates, we used different growth medium, which could explain the different urease activities, calcification rates and CO₂ sequestration capabilities of these isolates. In order to investigate the effects of the
different medium components on the CO₂ sequestration through MICP, we used the PB experimental design. PB design is a well-established and widely used optimization technique that allows the identification of environmental factors playing significant roles on microbial metabolisms (Stocks-Fischer, Galinat and Bang 1999; Bachmeier et al. 2002; Hammes, Boon and de Villiers 2003; Whiffin 2004; Sánchez-Román et al. 2007; De Muynck, De Belie and Verstraete 2010; Chou et al. 2011; Zambelli et al. 2011; Onal Okyay and Frigi Rodrigues 2014). In this study, the PB design allowed us to identify the significance of each medium component for each isolate and also assisted on the selection of the appropriate concentrations of each component to enhance CO₂ sequestration by each isolate. The PB results showed that urea was the most important component in all isolates’ growth media, which makes sense since urea is a key factor in ureolysis and all these isolates are ureolytic (Lee, Kim and Mahanty 2014). The PB results also allowed the enhancement of CO₂ sequestration, by the two worst ureolytic isolates, TR12 and CV1. These results suggested that CO₂ sequestration through MICP depends on the isolate itself and the microbial growth conditions. Further analyses of the biotic and abiotic effects on the CO₂ sequestration on these optimized media demonstrated that the optimization helped the microorganisms (TR12 and CV1) increase their CO₂ uptake metabolism to allow the enhancement of the biotic CO₂ uptake. Moreover, the change in the composition of the media led to higher abiotic CO₂ sequestrations in certain growth media. This higher abiotic sequestration was observed with growth media with more nutrients (or higher concentrations). This could be explained by the CO₂ diffusivity, which is dependent on the viscosity of the medium and temperature (Wilke and Chang 1955; McCabe, Maguire and Lintell 2005). Moreover, CO₂ solubility has been described to depend on environmental factors such as pH, temperature and pressure, and on intrinsic parameters such as salinity, sugar, fat and protein contents (Chaix, Guillaume and Guillard 2014). Hence, it is likely that by changing the medium composition, the viscosity of the medium changed and led to higher CO₂ uptake.

CONCLUSIONS

This study suggests that caves and travertines contain microorganisms involved in calcification processes and CO₂ sequestration. These findings also suggest that caves and travertines could be potential natural carbon sinks. Furthermore, calcifying bacteria were found to induce CO₂ removal from the atmosphere through two possible mechanisms: (a) biotic sequestration through MICP and (b) abiotic sequestration by changing the environment pH. In both cases, cells play significant roles in the CO₂ sequestration: first, they can act as nucleation sites for calcite precipitation; second, they can potentially sequester CO₂ by their fixation metabolisms; and third, they can increase the surrounding environmental pH, which would allow increasing atmospheric CO₂ dissolution in aquatic systems and calcite precipitation, when calcium is present. In this study, when considering the biotic and abiotic CO₂ sequestration mechanisms by MICP, the isolates, TR1, TR3, TR20, CV1 and TR12, were able to assist in the CO₂ sequestration by 78.6, 74.1, 63.6, 36 and 40% respectively, in their isolation media. Moreover, the PB design showed to be a powerful tool to determine the components and concentrations of each growth medium to significantly increase the abiotic CO₂ sequestrations for each isolate, and biotic CO₂ sequestrations for the isolates TR12 and CV1.

SUPPLEMENTARY DATA
Supplementary data is available at FEMSEC online.

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Conflict of interest. None declared.

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