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Vaterite bio-precipitation induced by *Bacillus pumilus* isolated from a solutional cave in Paiania, Athens, Greece

Markos I. Daskalakis [a], Fotis Rigas [a,*], Asterios Bakolas [a], Antonis Magoulas [b], Giorgos Kotoulas [b], Ioannis Katsikis [c], Aristomenis P. Karageorgis [d], Athena Mavridou [e]

[a] School of Chemical Engineering, National Technical University of Athens, 15700 Athens, Greece
[b] Hellenic Centre for Marine Research, Institute of Marine Biology and Genetics & Aquaculture, Heraklion, 71003 Crete, Greece
[c] Institute of Geology and Mineral Exploration, Athens, Greece
[d] Hellenic Centre for Marine Research, Institute of Oceanography, 19013 Anavyssos, Greece
[e] Technological Educational Institute of Athens, Department of Medical Laboratories, Athens, Greece

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ABSTRACT

In this work, *Bacillus pumilus* ACA-DC 4061 was selected for its increased capability for biomineralization on marble, under different nutrient media concentrations and temperature conditions. The optimum conditions for the $CaCO_3$ bacterially-induced precipitation were determined with the aid of testing based on the Design of Experiments (DoE). Biomineral (vaterite) precipitation was favored in both the temperatures (25 and 30 °C) investigated. Stone loss rate was reduced when the samples were subjected to sonication. Thin sections of the substrate confirmed that vaterite was able to adhere onto the surface. Finally, under non-sterile conditions mimicking an *in situ* application, *B. pumilus* ACA-DC 4061 formed a fine layer of calcium carbonate. Therefore, this microorganism showed that vaterite formation may consistently occur under specific conditions and could prove useful as a candidate for on-site applications for stone conservation.

Introduction

Looking back on Earth's history, we can appreciate the role played by bacteria in its evolution (Gadd, 2010). Recent scientific proof confirms the fact that bacteria had consistently participated in calcium carbonate deposition, thus improving the environmental conditions for further evolution of life (Zavarzin, 2002). Their great importance is presented by the ongoing research with reference to their remnants in the geological samples that may shed light on the planet's formation and history (Douglas, 2005). To date, substantial research is being conducted in order to interpret the processes of precipitation of the different calcium carbonate polymorphs due to their environmental and industrial importance. Besides understanding Earth's history and cave development (Barton and Northup, 2007), this knowledge also serves to promote the various sciences, including those of surgery and dentistry (Pecher et al., 2009), biomediated soil improvement (DeJong et al., 2010) and to improve the methods of CO_2 storage (Lee et al., 2010). Stone has found extensive use as a construction material, evident from the existing architectural monuments and statues. Nevertheless, the industrial revolution has accelerated the deterioration and degradation of several stone monuments through the emission of anthropogenic pollutants that interact with the physical (rain, air, sunlight) (Pope et al., 2002; Smith et al., 2008) and biological factors (Warscheid and Braams, 2000; Scheerer et al., 2009).

Previous research had focused initially on understanding the negative effects of bacterial proliferation upon different substrates and the correlation between the physical and microbial factors. Today, the beneficial features of bacterial metabolism are being investigated for stone protection, namely their ability to promote calcium carbonate precipitation under appropriate environmental and nutrient conditions (Boquet et al., 1973). Members of the *Bacillus* genus are among those more intensively investigated due to their ubiquity in nature.

One of the first microorganisms to be investigated in light of monument bio-restoration was the heterotrophic bacterium *Bacillus cereus* (Castanier et al., 2000). Based upon the patent applied (Adolphe et al., 1990), it was studied under specific nutrient

* Corresponding author.
E-mail addresses: fotios.rigas@gmail.com, rigasf@central.ntua.gr (F. Rigas).
URL: http://www.chemeng.ntua.gr

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conditions that provided the appropriate elements for calcium carbonate biomineralization. The methodology was evaluated in buildings and statues in France, and recently it was applied on plaster (Anne et al., 2010). One particular *B. cereus* strain isolated from a cave, has recently been reported to produce calcite under different nutrient conditions (Han et al., 2013).

Bacillus sphaericus possesses the advantage of the urease enzyme that, when selectively induced, promotes biomineralization (Hammes et al., 2003). Experimentation on limestone (Dick et al., 2006; De Muynck et al., 2013) and concrete (De Muynck et al., 2008a, b; Kim et al., 2013) revealed the potential of implementing this microorganism. *Sporosarcina pasteurii* (formerly *Bacillus pasteurii*), is another urease positive microorganism (Stocks-Fischer et al., 1999) tested primarily on concrete as an effective mixture component and for the bio-remediation of cracks. Results showed that although it did not provide satisfactory results for the first scenario, it could prove useful in shallow – crack remediation (Ramachandran et al., 2001). The latter, was further investigated with the application of immobilized cells in polyurethane foam (Bang et al., 2001) or premixed with sand and applied on cracks (Achal et al., 2013). A different approach to circumvent the mortality of *S. pasteurii* on concrete is the development of phenotypic mutants that can survive under conditions of high pH without the loss of ureolysis (Achal et al., 2009). The same microorganism was recently tested on bricks (Sarda et al., 2009).

Additional *Bacillus* species reported for their carbonatogenic activity are *Bacillus megaterium* (Cacchio et al., 2003), *Bacillus thuringiensis*, *Bacillus pumilus* (Baskar et al., 2006), *Bacillus pseudofirmus*, *Bacillus cohnii* (Jonkers et al., 2010), *Bacillus alkalinitrilicus* (Wiktor and Jonkers, 2011) and other *Bacillus* strains yet to be identified at the genus level (ChunXiang et al., 2010). Microorganisms from this genus have constantly been detected when selected media, which activate calcium carbonate biomineralization, were applied on quarried or decayed stone (Piñar et al., 2010; Ettenauer et al., 2011; Jroundi et al., 2012).

Investigation of the carbonatogenic ability of the isolates from stone or cave surfaces continues to remain of importance in order to understand the dynamics of the microorganisms and improve the growth media applied. Furthermore, the morphology of the biominerals deposited would assist in the differentiation between the inorganic and microbially induced biomineralization. Recently, bacterial isolates such as *B. cereus*, *Bacillus licheniformis* (Daskalakis et al., 2013) and *Cupriavidus metallidurans* ACA-DC 4073 (Daskalakis et al., 2014) were tested for their biomineralization capabilities. Similarly, we isolated and identified microorganisms isolated from a cave in Paiania, in the vicinity of Athens, Greece. One of the isolates, *B. pumilus* ACA-DC 4061, exhibited considerable biomineralization capability in the growth medium selected. Further investigations were done in order to delineate its potential to be applied on stone or exploited, if identified, in the stone microflora. Different media and growth parameters were investigated in order to detect the optimum bio-precipitation conditions and the endurance of the biomineral, which was mainly vaterite. Spraying the microorganism was tested under non-sterile conditions to partially mimic an *in situ* application and establish its potential and practicality. Finally, the statistical analysis presented and the biomineralization efficiency are discussed.

Materials and methods

Isolation, purification and genetic identification of Bacillus pumilus ACA-DC 4061

Samples for the current investigation were collected from a cave in Paiania district, near the city of Athens. The cave is accessible to visitors only during dry seasons. The following sample types were obtained: (i) cave wall material collected by sweeping the wall surface with sterile cotton swabs, which were subsequently immersed in nutrient broth; (ii) water dripping from stalactites immediately stored in sterile test tubes; (iii) non-coherent soil material from the sides of the stalagmites and (iv) water samples from a small pool of concentrated water percolating from the cave walls and stalactites hanging above. All the samples were obtained from sites far away from the visitors' walking path, lacking a direct light source and without an obvious chromatic difference. All the samples were stored immediately on ice for transport. Isolation and genetic identification were done as described previously (Daskalakis et al., 2013). The isolate, identified as *B. pumilus* ACA-DC 4061, was deposited in the Greek Bacterial Collection of Agricultural University of Athens – Greece – Laboratory of Dairy Research.

Growth media for biomineralization experiments

The basic growth medium implemented (BP1x) was 0.36 g of Bacteriological Peptone (LABM, Lancashire, UK) and 2.5 mL of 10% (w/v) calcium acetate solution [$(CH_3COO)_2Ca$ (Sigma–Aldrich, Munich, Germany)] in 100 ml deionized water. The growth media with 2x (BP2x) and 3x (BP3x) in the concentration specified above were also used during the biomineralization experiments. Liquid media were sterilized by autoclaving for 17 min at 15 psi – 121 °C. Screw-cap bottles of 250 mL capacity were filled with 100 ml of the growth medium. Qualitative observation of growth was performed by measuring the absorbance at 620 nm with a Hach DR-2000 spectrophotometer (HACH LANGE, Athens, Greece). The bacterial population (in CFU mL^{-1}) was calculated employing the serial dilution method.

Stone substrate

Marble from Penteli Mountain had been used in antiquity for several monuments and statues due to its high quality. Similar marble can be quarried only in the area of Dionysus (Daskalakis et al., 2013). Tiles made from this marble, were cut to sizes of 4 cm × 1 cm × 1–1.5 cm or 4 cm × 4 cm × 2 cm and used as the solid substrate.

Optimization of biomineralization factors

The biomineralization ability of *B. pumilus* ACA-DC 4061 was optimized through a series of tests based on the Design of Experiments (DoE). A Multilevel Factorial Design was used to construct a second order response surface with three design factors, namely, incubation temperature ($\boldsymbol{X_1}$), medium concentration as regards to bacteriological peptone – calcium acetate ($\boldsymbol{X_2}$) and inoculum concentration ($\boldsymbol{X_3}$). The design consisted of 15 trials replicated 5 times. The response selected under optimization was weight increase $\boldsymbol{Y}$ (g). The data of the statistical experiment were analyzed using the software Design-Expert 6.0.6 to determine the various components of the Analysis of Variance (ANOVA). The statistics that were used to determine whether the models, thus constructed, adequately described the experimental data included the significance of the models, lack-of-fit test and adequate precision statistic.

All the samples were analyzed for bacterial concentration, pH, calcium and acetate concentration, biomineral type and its morphology, as described below.

Optimal biomineralization experiment

The equation determined from the mathematical model mentioned above provided the optimal values regarding the