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Original Article

Application of Microbial Biotechnology in Conservation and Restoration of Stone Monument

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Abstract

Treatments employed for the consolidation of monumental stones made of limestone due to incompatibility from the substrate and cement used for consolidation, plugging of pores induced by the new cement, leading to the acceleration of stone alteration. Microbial precipitation with a layer of calcium carbonate generated by bacteria might offer a solution to this dilemma because the layer would not block the natural pore structure, thus permitting free passage of soluble salts through the stone. In this study, an attempt has been made to provide an overview of the microbial induced carbonate precipitation as promising technology for bioremediation of such structures. At the first, the active microorganisms in the conservation of stone monuments transferred to the laboratory using the swap dipped in nutrient broth at a historic cemetery. After incubation and growth of colonies, Gram-positive bacilli were detected. Then pure single colonies were transferred to blood agar medium and incubated at 37°C. the single colonies were transferred to the surface of sterilize limestone pieces and incubated but no result was obtained. Therefore, in the next phase bacilli bacteria-rich broth media was used. The control experiments were conducted in accordance with the conditions mentioned without bacterial inoculation. The calcification process caused by the inoculated bacteria on the historical stone samples was demonstrated using the scanning electron photomicrographs. Microbially induced calcium carbonate precipitation (MICP) technology to eco–friendly, self-healing and highly durable nature of these bio-binders, for conservation purposes has been found suitable. But still there has been much to explore in order to bring this environmentally safe, cost effective and convenient technology from lab to field scales.

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Introduction

Monumental stone decay is a consequence of the weathering action of physical, chemical and biological factors, which induce a progressive dissolution of the mineral matrix. Consolidation of structure of loose stones is the most important tasks of conservators. From the late 18th century that thought of preservation of original materials was formed, many researchers have been done regarding suitable material for saturation of monumental stone and a lot of organic materials such as oil, wax, resin, soaps, floating (hydrocyclofluoric acid salty solutions), water glass and silicic esters have been used for consolidation of stone [1]. Efforts to slow the destruction of historic stone using of protective treatments by organic or inorganic products, has created numerous obstacles and it has been proven none of them to create the protective conditions of damaged historic stone is not satisfactory.

Conventional treatments, both organic and inorganic, employed for the consolidation of ornamental stones have several disadvantages: long-term incompatibility from the substrate and the cement used for consolidation, plugging of pores induced by the new cement, all in all leading, in some cases, to the acceleration of stone alteration. It is now understood that application of a physical barrier on the stone surface will hinder the movement of salts, which can then build up, leading initially to unsightly discoloration and ultimately to physical damage. Even coatings that permit evaporation can cause problems of salt accumulation and crystallization; therefore, a protective coating must be sympathetic to the nature of the stone itself [2]. The most appropriate consolidating material is should be thoroughly infiltrated in the stone and don't just remain on the surface, especially if the stone is exposed to moisture and temperature changes [3]. Bioremediation is less harsh than the use of environmentally toxic chemicals or aggressive mechanical procedures, which are considered to be destructive methods. The production of the calcium carbonate layer generated by bacteria might offer a solution to this dilemma because the layer would not block the natural pore structure, thus permitting free passage of soluble salts through the stone [4]. Biomineralization is the process by which organisms form minerals, by creating physical and chemical conditions necessary for mineral formation and growth. Microorganisms are active in a wide range of mineralization processes and have been involved in the deposition of minerals throughout the history of the Earth [4]. Like other biomineralization processes, calcium carbonate biomineralization can occur by two different mechanisms: biologically controlled mineralization and biologically induced mineralization [5].

In biologically controlled mineralization, the organism controls the process (nucleation and growth of the mineral

particles) to a high degree. The mineral particles formed are synthesized or deposited on or within organic matrices or vesicles in a specific location with regard to the cell, and usually intracellular. Every organism synthesizes biogenic minerals in a form that is unique to that species, independently of environmental conditions. Because of these features, both the synthesis and the form of every specific biogenic mineral are thought to be under specific metabolic and genetic control. In contrast, biologically induced mineralization is usually carried out in an open environment, and no specialized cell structure or specific molecular mechanism is thought to be involved. Calcium carbonate deposition by bacteria is generally regarded as induced, and the type of mineral produced is largely dependent on environmental conditions. It is a very diffuse phenomenon and represents a fundamental part of the calcium biogeochemical cycle, contributing to the formation of calcium carbonate sediments, deposits, and rocks. Different mechanisms of bacterial involvement in calcification have been proposed, and they have been a matter of controversy throughout the last century. It is generally accepted that this microbial activity can be influenced by environmental physical-chemical parameters, and it is correlated to both metabolic activities and cell surface structures [6].

Saliva is the first biological fluid to encounter external factors including changes in eating habits and environmental or physical changes. The biochemical composition of saliva, as the first body fluid gating the gastrointestinal tract, is of prime importance. Various salivary markers may influences oral health both through its non-specific physico-chemical properties and specific effects [9]. Saliva is well known for its highly protective functions against deterious agents such as microorganisms, toxins and various oxidants [11]. The antioxidant capacity and reducing power of saliva is altered due to various factors including age [12] exercise [13-17] dietary supplementation [18, 19], food preservatives [20], internal diseases [21-24], smoking [25] and even passive smoking [26]. It has been shown that in vitro exposure to cigarette smoke could significantly decrease some enzymatic activities, both in plasma and in saliva [18, 27]. Due to the presence of valuable markers, non-invasive and easy sampling, saliva has recently attracted our attention to be used as a diagnostic body fluid [28, 29].

Our previous research activities were mainly based on the importance of saliva as a non-invasive biological fluid and its antioxidant alternations at a wide range of biological conditions. Therefore, the main aim of present research was to investigate alternations of some important non-antioxidant enzymes as well as the major salivary water soluble antioxidant, uric acid, in the oral fluid of subjects before, during and after the month of Ramadan fasting.

Materials and Methods

Media and sampling

The sample of bacteria was cultivated in 2 g/L yeast extract, 5 g/L peptone; beef extract 1 g/l and 5 g/L sodium chloride (pH 7). Bacterial strains were isolated from surface of historic limestone graves in separate cultures with the aim of finding active microorganisms in the conservation of stone works was done in the historic cemetery. Using nutrient broth at a historic cemetery and swab dipped in media, active microorganisms collected at the site and transported to the laboratory flask.

Isolation of single colonies

The samples incubate for seven days at 37ºC separately and then in order to isolation of single colonies were cultured on the blood agar plate and single colonies were isolated. The single colonies were stained by Gram-staining method in order to finding of the Gram-positive bacilli.

Inoculation of isolated bacteria into limestone samples

Limestone samples taken from the monuments with steam at 100ºC, four times were sterilized for 24 hours. Then, using multi-step culture method, pure colonies were obtained and transferred to blood agar plates. Finally isolated bacteria inoculated into sterilized samples of limestone and were incubated at 37ºC to growth bacteria and opacity of the culture medium. Also the bacilli bacteria-rich broth media was used. Control experiments were conducted in accordance with the conditions mentioned without bacterial inoculation [7]. To check the results of the tests and rate of Weight increase through biomineralization to conclusion regarding the considerable formation of bacterial-induced precipitation, weight changes was also measured. Limestone samples cultured and inoculated were recovered at different times. pH was measured both at the start of the experiment and when recovering the samples. Samples were rinsed three times with distilled water before drying at 37°C in a dark and dust-protected environment. Weight gain (average value of three samples) was calculated in terms of differences in weight between inoculated and control samples were determinate at the end of incubation.

Evaluation of ability of bacterial species for precipitation of calcium carbonate

To evaluation of ability of bacterial species for precipitation of calcium carbonate after growth of colonies and opaque of media, stone samples were dried up to death of bacteria. Calcified bacterial cell and biological precipitation of calcium carbonate on the porous substratum of limestone samples were observed by scanning electron microscopy (SEM) using a Philips XI30 SEM. Samples were coated with gold prior to observation. Both stone surfaces in contact with the bacterial media and sections perpendicular to the exposed surface were analyzed.

Results

We tested the ability of selected bacterial species for precipitation of calcium carbonate both on blood agar and liquid broth media. The samples were analyzed under an optical microscope to observe changes every day. The results of the morphological observation by SEM are shown in (Fig. 1). The stone surfaces in contact with the bacterial medium were analyzed. SEM observations showed amounts of calcified bacterial cells in Stone sample subjected to biomineralization (Fig. 2). Bacterial cells enclosed by calcium carbonate can be observed in samples treated with bacterial media. Calcified bacterial cells covered the walls of the pores after 30 days of culturing in the

bacilli bacteria-rich broth media. As per the second hypothesis of Biological mechanisms of calcium carbonate precipitation, carbonate nucleation takes place on the cell wall (Table. 1). The primary role of bacteria in this mechanism has been ascribed to their ability to create an alkaline environment through various physiological activities. Bacterial surfaces also play an important role, Due to the presence of several negatively charged groups, at a neutral pH, positively charged metal ions could be bound on bacterial surfaces, favoring heterogeneous nucleation.

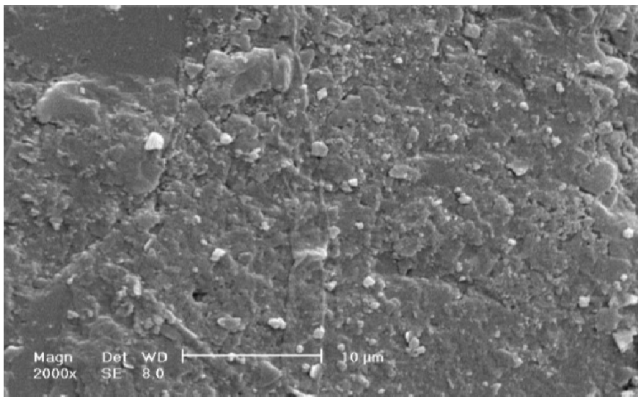

Figure 1. SEM Photomicrographs of limestone sample.

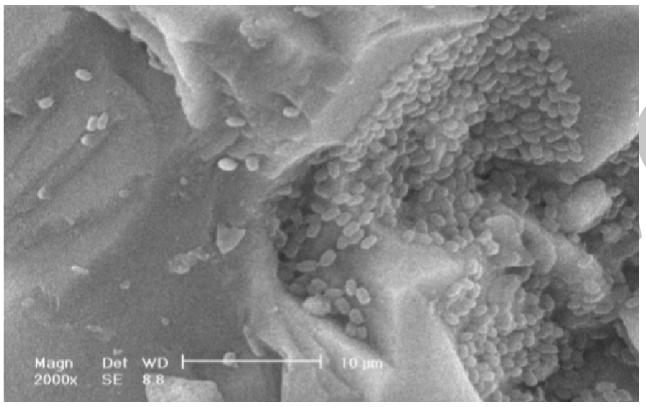

Figure 2. SEM Photomicrographs of limestone samples calcified by bacterial cells.

Discussion

In fact, surveys and studies about conservation of stone have shown that put thin and waterproof layer on the surface of stone not only protect it but also effect on deterioration/aging of monumental stones [7].The production of a calcium carbonate layer generated by bacteria might offer a solution to this dilemma because the layer would not block the natural pore structure, thus permitting free passage of soluble salts through the stone [8]. Also, different mechanisms as cause of formation of bacterial induced carbonate are reported, hypotheses related to the role of bacteria and their activities in carbonate crystallization can be summarized in the Table 1.

Boquet *et al*., (1973) firstly demonstrated the precipitation of calcium carbonate by soil bacteria under laboratory conditions. Previous researchers showed precipitation of carbonates by marine bacteria only in liquid media while Drew *et al*., (1911) and Shinano *et al*., (1972), investigated the carbonate precipitation by soil bacteria on solid media and obtained best results with B4 medium. Among the organisms tested, several Bacillus strains and Pseudomonas aeruginosa were observed to form crystals [14]. Castanier *et al*., (1999) reported the microbial origin of limestone while Adolphe *et al*., (1989) further demonstrated the bacterial origin of the calcite crusts and found great resistance against erosion by this calcite layer. Tiano *et al*., (1999) studied the effect of microbial calcite crystals on Pietra di Lecce bioclastic limestone by Micrococcus spp. and B. subtilis and their results showed a significant reduction in water absorption. Cappitelli *et al*., (2006) proposed Carbogel as a delivery system for bacteria due to its high retention of viable bacteria and less time to entrap cells. De Muynck *et al*., (2012) reported that *Bacillus sphaericus* is an efficient strain for consolidation of limestone specimens at range of temperatures (10, 20, 28, 37◦C). This isolate led to 64% lower weight loss upon sonication and 46% decreased sorptivity in treated limestone specimens compared to the control specimens [15]. De Muynck *et al*., (2011) recently applied bacterial calcite in two types of stones: microporous and macroporous. They reported that application of bacterial carbonates is more successful in macroporous stone where it occurs to a larger extent and at greater depths than in microporous stone. From the above mentioned applications, microbial concrete seems to bring a new revolution in the civil industry. Use of bacteria to improve the durability of building materials has drawn the attention of research groups all over the world. But several challenges have to be met before acceptance of this technology by conservators [16].

In natural conditions, the precipitation of $CaCO_3$ (carbonatogenesis) can be considered as the result of a series of chemical (abiotic precipitation) and biochemical processes (biotic precipitation). The equilibrium exists between insoluble carbonate and soluble bicarbonate forms in the water (Eq. 1). The depletion of CO_2 from water favors the deposition of carbonate [9].

$$Ca^{++} + 2HCO_3^- <=> CaCO_3 + CO_2 + H_2O \quad (1$$

Abiotic chemical precipitation can occur due to a decrease in the partial CO2 pressure, shaking or stirring of water, an increase in temperature, or a decrease in hydrostatic pressure. Biotic precipitation is due to different bacterial species that precipitate carbonate in alkaline environments rich in calcium ions [9].

One of the most relevant and well-known examples of mineralization driven by bacteria is the precipitation of-$CaCO_3$. This group of microorganisms is known as carbonatogenic microorganisms or calcifying microbes, because of their inherent capability of producing calcium carbonate [10]. In our experiments also bacilli colonies inoculating broth media as calcifying microbes covered the pores of historical limestone samples because of their ability to create an alkaline environment by physiological activities. Due to combination of medium and conditions of culture, weight gain resulting from this phenomenon in inoculated samples did not change significantly. Because it has been

proven that rate of precipitation of calcite as microbial induced on suitable media supplemented with a calcium source, has been more [2] and so the composition of the culture medium and culture conditions are key factors for increasing the effectiveness of protective and consolidate new formed carbonate in limestone structure is loose [12]. So if the medium supplemented with a calcium source used was obtained better results. It seems that bacterial calcification in inoculated limestone samples is as the product of microbial metabolism so that calcium carbonate precipitations on the outer surface of bacterial cells are grown, and bacteria embedded in growing carbonate crystals.

structures. In comparison to chemical treatments, because the microbial activity depends on many environmental factors, this type of method used is found to be more complex. As above-mentioned consolidating and/or protecting effects, carbonate cementation to a depth of several hundred micrometers and no plugging or blocking of pores takes place during this cementation, but some negative consequences, such as the formation of new products due to chemical reactions between stone minerals and some by-products originating from the metabolism of bacteria, and the formation of stained patches because of the growth of air-borne fungi, can be observed [12, 5].

Table 1. Biological mechanisms of calcium carbonate precipitation.

	How to formation of calcium carbonate	Type of microorganism involved	process
First hypothesis	mineralization occurs as by-product of microbial metabolism involving either autotrophic or heterotrophic pathways	(i) photosynthetic organisms such as cyanobacteria and algae, (ii) sulfate reducing bacteria responsible for dissimulator reduction of sulfates, (iii) organisms utilizing organic acids, and (iv) organisms that are involved in nitrogen cycle either by ammonification of amino acids/nitrate reduction or hydrolysis of urea (5)	Heterotrophic bacteria several pathways from the nitrogen and sulphur cycle are involved in carbonate precipitation. In these processes, reaction, such as enzymatic hydrolysis of urea or disassimilatory reduction of nitrate and sulphate, cause an increase in pH, which shifts the carbonate-bicarbonate equilibrium towards the production of more carbonate ions and, ultimately, the precipitation of calcium carbonate, if free calcium ions are present pH [6]. In general, metabolic pathways able to increase the environmental pH toward alkalinity can, in the presence of calcium ions, foster calcium carbonate precipitation. Due to the presence of several negatively charged groups, at a neutral pH, positively charged metal ions could be bound on bacterial surfaces, favoring heterogeneous nucleation. Commonly, carbonate precipitates develop on the external surface of bacterial cells by successive stratification, and bacteria can be embedded in growing carbonate crystals [2].
Second hypothesis	carbonate nucleation takes place on the cell wall		The first step is a stoichiometric interaction of metal with reactive chemical groups, present on wall cellular: after complexation, these sites nucleate the deposition of more metal as a chemical precipitate. Commonly, carbonate precipitates develop on the external surface of bacterial cells by successive stratification and bacteria can be embedded in growing carbonate crystals [6].
Third hypothesis	role of extracellular macromolecules such as extracellular polymeric materials produced by bacteria		Proteins present in biological extracellular polymeric materials may cause the formation of different and polymorph CaCO3 crystals [6].

In a similar experiment by Barabesi *et al*., about the search for *Bacillus subtilis* gene cluster involved in calcium carbonate biomineralization using Colonies of six different strains of *Bacillus sphaericus* and *Bacillus lentus* on agar plates, their ability to encrust themselves in calcium carbonate was proved [2]. In SEM photomicrographs of limestone cultivated in the M-3 medium and inoculated with *Myxococcus xanthus* was performed by Rodriguez-Navarro *et al*., too, calcified bacterial cells covering the pore walls of samples (after 30 days) has been shown [12]. On the other hand, in situ small-scale testing of the method we used in monumental calcareous stones will be difficult and should be performed before larger-scale applications are undertaken in buildings and sculptures.

Despite some limitations, there are many advantages of this technology for bioremediation of Calcareous stone

It has already been demonstrated that uncontrolled bacterial growth can damage stone. For this reason, development of a stone treatment without viable cells (Unlike our experience) seems a better biotechnological tool. Some studies show that extracellular polymeric secretions influence calcium carbonate precipitation in a positive way. In this cellular fraction some proteins are over expressed when calcium ions are present in the cultural media. Study of these proteins will help in cloning their genes thus facilitating identification of the functions. Further investigation should be directed to the isolation and purification of the protein responsible of bio precipitation. Further researchers are in progress directly aiming to develop biotechnological processes by which stone decaying surfaces can be restored utilizing proteins isolated from bacteria without living cells. This avoid that some bacterial metabolite,

excreted by living cells, can negatively influence the restoration [10, 12]. Extracellular polymeric materials such as exopolysaccharides (EPS) and capsular polysaccharides (CPS) produced by microorganisms may be tightly bound to the cell (cell attached or capsular) or loosely adherent to cells (slime type, free, or released) or in the form of free dissolved material. Many bacteria may be surrounded by a polysaccharide containing outer layer termed glycocalyx. When this layer is tightly bound and remains attached to cells, it is referred to as a capsule. In more research of biomineralization of calcium carbonate, a study with the aiming looking for cell structures of bacteria involved in calcite crystal formation, outer structures (EPS and CPS) from *Bacillus firmus* and *Nocardia calcarea* were isolated to check on their influence in calcite precipitation. Results showed that both strains produce mucoid colonies that precipitate calcite crystals coated with thick "mucilage" [12].

Plugging of the pores is mainly a consequence of EPS film formation. This film has sometimes been misinterpreted as a newly formed carbonate deposit [12]. If precipitation time increases, then the amounts of EPS production increases, and hence plugging but multiple applications of nutrients and usage of carrier materials have significant influence on the total cost of treatment [13], but it has been demonstrated that culture medium composition, culture conditions, and type of bacteria are key factors for controlling EPS film formation and for the enhancement of the consolidation and protection effects of the newly formed carbonate cement [12].

Microbially induced calcium carbonate precipitation (MICCP) is found to be more complex because depends on factors such as temperature, pH, concentrations of donors and acceptors of electrons, concentrations and diffusion rates of nutrients and metabolites. Design of experiments for biodeposition treatments require a huge data of the biological processes (growth, biosynthesis, specific enzymatic activities), chemical reactions accompanied with formation of insoluble compounds, physical and chemical processes as precipitation, crystallization, and adhesion. Due to this complexity, its usage at large-scale has not been so encouraging. Additional research is necessary to overcome this problem. As the amounts of carbonate precipitates formed are dependent on amount of calcium added, increased concentration of calcium leads to accumulation of salts and paves way to efflorescence and damage to crystallization. The survival of bacteria within the stone material also influences the extent of calcification. Large scale production of bacterial cultures is also a hindrance in the path of success of this technology over traditional treatments [12, 13]. It has also been demonstrated that uncontrolled bacterial growth can damage stone. For this reason, development of a stone treatment without viable cells seems a better biotechnological tool [12]. There is need to assess the long term efficacy of microbial carbonates and compared to chemical binders. The success of this technology needs experts from varying field's including microbiologists, geologists and civil engineers. Researchers from all around the globe should work together to make this multi-disciplinary research move toward commercial scale applications at a higher pace [13].

Conclusion

Biomineralization induced by bacterial activity has significant protection and consolidation of porous carbonate stones which can be used in sculptural and architectural heritage. However, much research has yet to be conducted in order to properly exploit this new conservation methodology.

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