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Review

Bioconservation of Historic Stone Buildings—An Updated Review

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Abstract: Cultural heritage buildings of stone construction require careful restorative actions to maintain them as close to the original condition as possible. This includes consolidation and cleaning of the structure. Traditional consolidants may have poor performance due to structural drawbacks such as low adhesion, poor penetration and flexibility. The requirement for organic consolidants to be dissolved in volatile organic compounds may pose environmental and human health risks. Traditional conservation treatments can be replaced by more environmentally acceptable, biologically-based, measures, including bioconsolidation using whole bacterial cells or cell biomolecules; the latter include plant or microbial biopolymers and bacterial cell walls. Biocleaning can employ microorganisms or their extracted enzymes to remove inorganic and organic surface deposits such as sulfate crusts, animal glues, biofilms and felt tip marker graffiti. This review seeks to provide updated information on the innovative bioconservation treatments that have been or are being developed.

Keywords: biocleaning; bioconsolidation; biocalcification; biopolymers; carbonatogenic bacteria; cultural heritage; MICP; microorganisms; stone restoration

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1. Introduction

Our stone cultural heritage is subject to weathering over the years. This deterioration is due to physical, chemical and biological factors acting synergistically that cause disfiguration and dissolution of the stone, an increase in porosity and overall weakening of the structure [1]. When the building is of historic and cultural importance, it is necessary that careful restorative action be taken, with the aim of returning the monument, as much as possible, to its original condition. This may involve removal of unwanted surface deposits (cleaning) and strengthening of degraded parts of the structure (consolidation), and ultimately replacement of blocks or whole sections of the structure. Several processes, such as microbial transformation of stone minerals [1], discoloring by microbial pigments including algal, bacterial and fungal biomolecules with staining properties [2–5], and atmospheric pollutant deposition [6], which may all operate simultaneously at surfaces, can make cleaning procedures challenging. Furthermore, cleaning can induce irreversible damage; thus a cost-benefit analysis should be considered in a prior assessment phase, in order to reduce potential risks to artwork [7].

The consolidation and conservation of these buildings requires the use of materials that are compatible with the original structure. Consolidants that have been used in the past, or, indeed, are still in use, include those based on lime water, which is not very efficient [8], and silicic acid, which can lack the necessary flexibility for resisting stresses within the stone [9]. Nanoconsolidants, used as recent improvements in consolidation strategies [10] include the use of nanolime [11] and nanosilica and may be more effective in reducing porosity [12,13]. Nanolime doped with calcium hydroxide/zinc oxide quantum dots has been shown to form a more durable conservation treatment for limestone than silica-based consolidants [14], but it is more costly. The disadvantages of traditional consolidants, for

both calcareous and siliceous stone, are poor performance, structural drawbacks such as low adhesion, poor penetration and flexibility and, in the case of organic substances such as alkoxysilanes, the necessity to be dissolved in volatile organic compounds (VOCs), which carry environmental and human health risks [15].

Synthetic resins, such as silane, epoxy, acrylic and polysiloxane, plug the pores when they polymerize, thus causing water retention and internal degradation, while external coatings can peel off. In order to increase the efficiency, several chemical modifications of old consolidants have been produced [16]. However, perhaps more promising are the new, biologically-based consolidants (bioconsolidants), which do not rely on synthetic components, but are naturally-occurring structural plant biopolymers [17,18], or utilize the enzyme activities of microorganisms to produce new materials.

Not only consolidants, but other traditional conservation treatments, can be replaced by more environmentally acceptable, biologically-based, measures to restore stone. This review seeks to provide updated information on these innovative conservation treatments that have been or are being developed.

2. Bioconsolidation Techniques

According to Wheeler [19], a consolidant is a material, or system of more than one material, that penetrates a substratum, improving the inner structure and thereby enhancing the mechanical properties, thus improving the adhesion of surfaces. A good consolidant needs to meet three criteria: (i) compatibility (not causing chemical or physical damage) with the original substratum; (ii) effectiveness by penetrating evenly within the inner structure, and (iii) durability by not yielding noxious by-products after application. Recently, Negri et al. [20] also stressed the need to meet the retractability criterion by not interfering with previous interventions.

Limestone and lime-based mortars have been consolidated using plant biopolymers. Reported biopolymers have been sourced from *Aloe vera*, *Cylindropuntia californica*, *Opuntia engelmannii*, *Opuntia ficus-indica*, *Salvia hispanica* and *Sida angustifolia* [17,21]. Biopolymers are obtained in a range of extraction procedures, soaking leaves and other leafy tissues in batches of water and subsequently leaving them to be released into the bulk water. This water containing the biopolymers is then used for mixing with lime and other materials and used for consolidation or used as a suspension. Consolidants are then applied to the stone surface using techniques such as spraying, brushing or partial immersion, or mixed with other materials to yield composites [22]. Biopolymers alter mechanical properties of building materials and their water absorption and diffusion behavior, thus increasing cohesion and enhancing mechanical properties [23]. Bioconsolidation is also achieved by direct or indirect application of microorganisms and/or their metabolites [24]. Microbially induced carbonate precipitation (MICP), also known as biocalcification, biocarbonatogenesis, or biomineralization, has been suggested as an environmentally friendly method for the consolidation of cultural heritage buildings. It is a natural phenomenon, induced by a wide variety of microorganisms, that can be responsible for chalking (the production of a white, powdery surface) [25], as well as playing an essential role in the formation of stromatolites [26]. This microbially mediated mineralization treatment must produce a coherent calcium carbonate layer that can protect deteriorated stone against water uptake and consolidate its inner structure. Such a natural process should result in a mineral product similar to the initial calcareous stone substrate, a highly desirable result in the eyes of conservators.

There have been several strategies: the application of selected carbonatogenic bacteria to the stone, enrichment of naturally occurring carbonatogenic bacteria, application of cell-free bacterial products, and stimulation of the relevant microorganisms among those already present (autochthonous microorganisms). There have been a number of reviews covering these options in recent years [26–31]. In this bibliographic search, we have attempted to avoid the sometimes lengthy discussions of the history behind the application of the technology and its variants, along with the minutiae of the treatments, preferring to

reference such studies and using Table 1 to indicate relevant aspects of the developmental research. We have also considered the less frequently discussed environmental impacts of the new methods; only very recently have the effects on the normal microbial communities of the stone surfaces, and the resistance of the restoration to colonization by potential deteriogens been considered. A perspective on the development of biotechnological sustainable products for ultimate long-term use is also included.

2.1. Microbial Cells in Bioconsolidation Treatments

When considering the application of selected bacteria, a good option is to use organisms isolated from the autochthonous population, which are obviously already adapted to the stone environment and are less likely to interact negatively with the endogenous community [24,32,33]. The first such bacterial strain to be so isolated was a *Bacillus cereus*, which produced carbonate crystals in the alkaline conditions caused by breakdown of amino acids to ammonia [34]. It was successfully tested in the field in 1999 (see Table 1 [35]).

Table 1. Examples of carbonatogenic microorganisms that have been tested for MICP potential.

Organisms	Isolation Source/ Habitat	Testing Method	Reference
Bacillus, *Pseudomonas*, *Brevibacterium*, *Streptomyces*, *Stenotrophomonas* genera	Degraded limestone monument (Romania) Isolated during the course of the study	Crystal formation by isolated strains on solid medium; crystal identification by FTIR, XRD and SEM	[36]
Acinetobacter sp.	Cave (Yixing Shanjuan, China) Carbonate stone (Spain) Isolated during the course of the study	Consolidation of artificial cracks in masonry cement mortars; compressive strength, water absorption, SEM, XRD, thermogravimetry In vitro consolidation with *Mixococcus xanthus;* chemical analysis of calcarenite stone	[37] [32]
Agrococcus jejuensis sMM51 (Soil, Middle Muschelkalk)	Limestone-associated groundwater, rock and soil (Germany)	Crystal formation in liquid culture; crystal morphology by XRD, EDS and SEM	[38]
Bacillus muralis rLMd (Rock, Lower Muschelkalk) *Bacillus* sp. rMM9 (Rock, Middle Muschelkalk)	The strains are deposited with the Jena Microbial Resource Collection (Jena, Germany)		
Bacillus subtilis 168 (®27370)	ATCC: American Type Culture Collection, Manassas (VA) USA	Crystal formation on solid medium; detailed crystal analysis	[39]
Indigenous bacterial community	Salt-damaged carbonate stone in San Jeronimo Monastery, Spain. Isolated during the course of the study	Consolidation of originating monument; drilling resistance, SEM	[24]
Synechococcus pevalleikii (live and dead)	National marine laboratories, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India	Concrete cubes in vitro. U-V treated cells gave better compressive strength and lower water uptake	[40]
Synechocystis (6803)	PCC: Pasteur Culture Collection (Paris), France	Morphological and spectroscopic changes of mortar surfaces	[41]
Gloeocapsa (73106).	PCC: Pasteur Culture Collection (Paris), France	Measurement of compressive strength, water absorption and porosity of treated mortar	[42]
Pseudomonas sp. (N9), *Bacillus cereus* (T6), *Lysinibacillus sphaericus* (T5), *Bacillus* sp.	Historic white marble (China) Isolated during the course of the study	Crystal formation in solid and liquid culture by XRD analysis	[43]

Table 1. *Cont.*

Organisms	Isolation Source/ Habitat	Testing Method	Reference
Bacillus sp., *Micrococcus* sp.	Black crusts on limestone buildings Isolated during the course of the study	Bio-cementation on limestone slabs; Bio-consolidation of fragmented stones (Church of Santa Maria dei Miracoli, Venice); SEM, EDX	[44]
Bacillus cereus	Natural carbonate rock Isolated during the course of the study	First in situ consolidation attempt (Saint Médard Church, Thouars, France); SEM, water absortion, surface roughness, colorimetry	[45]
Bacillus subtilis LMG 3589	Belgian Co-ordinated Collections of Microorganisms	Consolidation of deteriorated *Globigerina* limestone in Malta; drilling resistance, water absorption, salt deterioration, porosity	[46]
Lysinibacillus sphaericus, *Bacillus subtilis*, *Pseudomonas putida*	INQCSS: Instituto Nacional de Controle de Qualidade em Saúde. Brazilian Culture Collection (Rio de Janeiro, Brazil)	Production of $CaCO_3$ in growth medium	[47]
Pseudomonas, *Pantoea*, *Cupriavidus*	Ancient marble quarry in Athens, Greece Isolated during the course of the study	SEM, ERD and FTIR analyses of treated marble from same quarry	[48]
Psychrobacillus psycrodurans	Mortar and concrete samples (National University of Colombia—Bogota, buildings) (IBUN: Institute of Biotechnology of the National University of Colombia. Collection of microorganisms (Bogotá, Colombia))	Biocementation tests on mortar cubes; SEM, XRD, compressive strength	[49]
B. licheniformis DSMZ 8782, *B. cereus* 4b, *S. epidermidis* 4a, *M. luteus* BS52, *M. luteus* 6	DSMZ: German Collection of Microorganisms and Cell Cultures GmbH (Brunswick, Germany) Collection of the Enzymology laboratory of B.P. Konstantinov Petersburg, Nuclear Physics Institute NRC "Kurchatov Institute" (Moscow, Russia)	Repairing microcracks in cement, calcium carbonate precipitation	[50]
Sporosarcina pasteurii	DSMZ: German Collection of Microorganisms and Cell Cultures GmbH (Brunswick, Germany)	3-D printing using sand; hardening tests	[51]
Pseudomonas (isolates D2 and F2) and *Acinetobacter* (isolate B14)	Freshwater sessile bacteria Isolated during the course of the study	Histological and fluorescence staining determination of cell viability inside carbonate crystals, and pore size reduction in limestone by image analysis.	[52]

Table 1. *Cont.*

Organisms	Isolation Source/ Habitat	Testing Method	Reference
Penicillium chrysogenum CS1 (Cement Sludge)	Isolated from Cement sludge Isolated during the course of the study	Cementation in sand column to form sandstone; compressive strength	[53]
Colletotrichum acutatum	Diseased fruit crops Isolated during the course of the study	Inoculation on limestone; SEM, EDX, XRD	[54]
Paecilomyces inflatus, *Plectosphaerella cucumerina.*	Stalactite growing from a concrete ceiling Isolated during the course of the study	SEM and XRD of carbonate crystals associated with hyphal growth in broth	[55]

Abbreviations: FTIR: Fourier transform infra-red spectroscopy; XRD: X-ray diffraction analysis; SEM: scanning electron microscopy; EDS: energy dispersive X-ray spectroscopy.

One published example is that of Micallef et al. [45], who effectively treated deteriorated limestone with biocalcifying *B. subtilis* isolated from Maltese hypogea. The treatment conferred uniform bioconsolidation to a depth of 30 mm and the treated stone had high resistance to salt deterioration and low water absorption, with a preserved pore network. Further work on this technology is still necessary. Research by one of the authors is currently underway on a limestone wall in Campeche, southern México (Figure 1). Bioconsolidation of the damaged limestone blocks is underway using a suspension of a locally isolated carbonatogenic bacterial strain applied by spraying. Frames have been set to study the efficiency of consolidation in several quadrants, to determine variability of the results. Efficiency is being measured by surface hardness and cohesion, using peeling tests. Preliminary measurements made two months after application revealed a slight increase of surface hardness and less flake detachment. The experimental study will be continued for up to one year and both short- and medium-term effects of the treatment will be determined under the natural climatic conditions of this region, classified as Köppen climate As (Tropical Savannah).

Many other microorganisms have been tested for their calcifying and consolidation activities, including fungi (see Table 1). An important proportion of microorganisms reported in Table 1 are well characterized isolates that are deposited in established culture collections, while a significant number of bacteria were isolated during the course of the respective studies. The latter implies they are not accessible to other research groups for reproducibility or to undertake new studies. This is important for further research and also in terms of intellectual property rights. Access to microbial strains possessing desired properties such as biocalcifying activity can be exploited by patented biotechnological processes.

Chuo [55] suggested that the most studied bacterium for MICP over the years was *Sporosarcina pasteurii*. Omoregie et al. [56] found that this genus was the most abundant biocalcifying organism isolated from limestone cave samples, but many other species have been isolated, both by these authors and others (for example, [57]); since microorganisms with this ability are common, it is clear that there is a wide range of species available from which to choose the most appropriate for the particular stone and environment. The stone environment is often considered oligotrophic and thus isolation strategies of lithic-dwelling microorganisms should consider the design and use of low nutrient media [58,59].

Figure 1. Limestone wall in Campeche, southern México, exhibiting a range of deterioration pathologies. Formers are being used to demarcate panels prior to treatment by spraying with carbonatogenic bacterial suspension or control solution. Replicate panels will allow statistical analysis. The aim is to increase surface hardness and reduce the detachment rate of flaking material.

Not only is the type of culture medium an important factor in the design of strategies aiming to retrieve a large number of relevant bacteria from environmental samples, but it is also necessary to use a range of isolation methods, including plating, enrichment and micromanipulation or extinction-culturing, either alone or in combination [60]. This combined approach is imperative to obtain more pure cultures of potentially dominant microorganisms derived from the stone environment, allowing a significant advance in the study of the physiology of carbonatogenic microorganisms and the environmental determinants of their activity and abundance. Pure physiologically characterized novel bacterial isolates are a prerequisite for biotechnological development across economic sectors and markets, ranging from those traditional ones already used in the food or pharmaceutical industries to emerging sectors such as cultural heritage protection.

The relatively new techniques of metagenomics and metabolomics could also play a role in selecting suitable organisms. Chimienti et al. [61], using next generation sequencing (NGS), identified the presence of known carbonatogenic bacteria like *Arthrobacter* in the populations colonizing the medieval church of San Leonardo di Siponto in Italy. 16S rRNA and functional gene analyses could show the carbonatogenic potential of metabolic pathways linked to the appropriate biogeochemical cycles. For metabolomics to be successfully applied to the discovery of new potentially useful organisms, however, it is necessary to better understand the biochemical processes involved in carbonatogenesis. Earlier genetic studies have, for example, used mutants impaired in $CaCO_3$ precipitation to indicate a link between biomineralization, fatty acid metabolism, altered phospholipid membrane composition and surface properties [8,31,62–64]. Fatty acid metabolism has also been implicated by work that showed how $CaCO_3$ precipitation in *Lysinibacillus* could modify membrane rigidity by upregulating branched chain fatty acid synthesis [65,66].

The prospects for the future are promising, with the combined use of improved culturing methods and metabolomics-based selection of novel carbonatogenic species. The study of their physiology and ecology should enhance the success of bioconsolidation strategies, in particular for long-term application.

Of course, other factors affect biocalcite precipitation, apart from the particular microbial species. These are cell and nutrient concentrations, source of calcium, presence of other substances, such as surfactants used as dispersants [55] and salt [24], and methods used to inoculate the bacteria onto the stone surfaces. There are several publications around this topic [31,67–69].

The alternative to application of selected carbonatogenic bacteria is to try to stimulate those already present on the stone surface. Jimenez-Lopez et al. [70] tested this approach in vitro, immersing porous limestone slabs in a nutrient medium. The positive result obtained was shown to be due to ammonification of the medium. Later, a similar approach was adopted by Jroudi et al. [71] for the consolidation of badly degraded tuff stone and lime plaster at the Mayan site of Copan (Honduras). The stone was treated with patented (sterile) M-3P nutrient solution containing amino acids and calcium. This resulted in significant changes in the indigenous bacterial community, which were detected using NGS techniques. There were increased levels of *Arthrobacter*, *Micrococcaceae*, *Nocardioides*, *Fictibacillus*, *Streptomyces* and *Rubrobacter*. In the lime plaster, *Bacillus*, *Agrococcus*, and *Microbacterium* were the major genera after treatment. It is notable that most of the increased components of the community after treatment were capable of calcium carbonate biomineralization. Although this may indicate that the treatment could replace application of living microorganisms, the detection of biocalcifying microorganisms does not necessarily imply that these will produce a good consolidating performance, and further testing, such as that suggested in Table 2 below, would be necessary.

Table 2. Properties of representative consolidants and methods of evaluation for stone conservation [72,73].

Consolidating Properties	Consolidation Action	Substrate	Consolidation Treatments	Evaluation of Treatment
Effectiveness	Penetration depth	Marble, limestone, and lime-based mortars	Phosphate treatment based on Hydroxyapatite (HAP) Ethyl silicate (ES)	Scanning electron microscopy (SEM)
Compatibility	Morphology and microstructure of samples resulting from applied products	Marble	Nano-solution of calcium tetrahydrofurfuryloxide (Ca (OTHF)2)	Optical Microscopy (OM) and Scanning Electron Microscopy coupled with Energy Dispersive X-ray spectroscopy (SEM-EDX)
Effectiveness	Mechanical properties: compressive strength, tensile strength, bending strength, modulus of elasticity, ultrasonic pulse velocity, abrasion loss, surface hardness.	Marble, limestone, and lime-based mortars	HAP ES	For example, tensile strength (σ_t) by the Brazilian splitting test using an Amsler-Wolpert loading machine

Table 2. *Cont.*

Consolidating Properties	Consolidation Action	Substrate	Consolidation Treatments	Evaluation of Treatment
Effectiveness	Hardness of the substrate; Increase in mechanical strength; penetration depth of treatment	Limestone/lime based mortar	Nanolime	Drilling Resistance Measurement System (DRMS)
Compatibility	Color changes induced by the treatment	Limestone/ Marble	HAP/nano-solution of calcium tetrahydrofurfuryloxide (Ca (OTHF)2)	Spectrophotometer Konica Minolta CM-700d to measure CIEL*a*b* coordinates
Compatibility	Newly formed phases and secondary by-products	Marble, limestone, and lime-based mortars	HAP ES	Fourier transform infrared spectroscopy (FT-IR)
Compatibility	Microstructure: variations in open porosity and pore size distribution after the treatment	Marble, limestone, and lime-based mortars	HAP ES	Mercury intrusion porosimetry (MIP)
Compatibility	Thermal behavior: samples subjected to thermal cycles (heating–cooling cycle)	Marble, limestone, and lime-based mortars	HAP ES	Use of dilatometer L75/30/C/W Ceramic Instruments
Durability	Accelerated weathering cycles: Wetting–drying, freezing–thawing and salt crystallization cycles	Marble, limestone, and lime-based mortars	HAP ES	European EN 12371 and Italian UNI 11186 for freeze–thaw test, European EN 12370, RILEM MS-A.1 and RILEM MS-A.2 for salt weathering

2.2. Microbial Products in Bioconsolidation Treatments

Perito et al. [74] showed that dead, as well as live, *B. cereus* cells could produce carbonate crystals in liquid medium, the cell bodies acting as crystallization nuclei. It is not necessary to have whole cells for this; bacterial surfaces such as cell walls or extracellular polymeric substances (EPS) have metal binding properties that can also serve as nucleation sites [75–77]. The ability of cell walls to uptake cations such as Ca^{2+} was demonstrated directly for *B. subtilis* in much earlier work [78].

The advantages of non-cell treatments are that nutrients and appropriate growth conditions are not required and that cell components are smaller than whole cells and therefore able to penetrate further into cracks and pores (but, of course, without clogging the latter). This type of product is, however, more difficult and more expensive to prepare. A mixed inoculant, containing both EPS and living carbonatogenic cells, could give an improved performance [79].

EPS have fundamental cellular functions, including attachment to surfaces and provision of a framework for architectural growth in biofilms. They possess a varied chemistry; the composing biomolecules may include proteins, polysaccharides, lipids plus nucleic acids, lipids alone, and uronic acid. EPS may inhibit or enhance precipitation of calcium

carbonate, depending on the functional groups which enhance precipitation by serving as initial nucleation sites, such as anionic groups like sulfate and acidic sugars [80]. Microbially influenced precipitation occurs due to the interactions of extracellular biopolymers and the geochemical environment. This is seen, for example, in the production of moonmilk speleothems, natural calcium carbonate deposits in caves, which has been associated with filamentous actinomycete activity through metabolic profiling of biomineralization pathways [81]. This, indeed, could be an interesting option for further research into bioconsolidation.

There is no consensus on the optimal conditions for biocalcification treatments, regardless of the actual method used, whether with or without cells [82]. This may be due to the fact that optimum conditions will vary depending on the type of stone, the geometry of the structure to be treated and the climate in the particular geographic location.

Conditions will certainly vary depending on the type of stone to be consolidated. Pore structure affects penetration depth and treatment performance [81]. The polymorphs produced by biocalcification are mainly calcite (rhombohedral), aragonite (needles) and vaterite (hexagonal), the final form depending on environmental conditions and bacterial strains. The most common precipitated forms are calcite and vaterite [83–85], calcite being the dominant and most thermodynamically stable [86,87], although it has been suggested that microbially-induced vaterite achieves similar stability to calcite through the incorporation of organic molecules [68,88]. Hydrated phases of $CaCO_3$ have also been reported [89,90]. The nanomechanical properties of the $CaCO_3$ polymorphs could be improved by increased understanding of the biogeochemical processes involved [91].

3. Effectiveness of the Consolidated Stone in the Environment

It is important to know how the new material will perform in its particular environment. Various test methods have been used to evaluate consolidation (Table 2).

In comparison to synthetic consolidants, such as those shown in Table 2, few studies have characterized the performance of bioconsolidation methods; this is an avenue of research that could allow the development of novel approaches and strategies integrating microbiological agents or their metabolites. Novel consolidants need not only to be assessed in terms of the properties shown in Table 2, but also in their response to any microbial insults from the immediate environment.

It is imperative that the effects of the environment on the new consolidant are considered, in order to assess consolidating integrity and durability. This deserves particular attention in the future, necessitating lengthy commitment to such long-term studies.

Biodeterioration Testing and Colonization of Biocalcite

There have been few studies on the colonization of the newly produced material by environmental microorganisms, although a recent article reviews the methodologies employed to assess the durability of cultural heritage stone surfaces in response to microbial colonization [92]. Recolonization after the use of non-biological consolidants on marble, sandstone and plaster in the archeological site of Fiesole, Italy, was shown to depend mainly on the bioreceptivity of the substrate and the climatic conditions [93]. A relatively recent paper suggests that the bioconsolidation treatment based on stimulation of indigenous calcifying microorganisms does not significantly alter the stone microbiota in the long term [94]. The authors showed, using DGGE monitoring of the stone-autochthonous microbiota before and at 5, 12 and 30 months after bioconsolidation with autochthonous bacteria, that the *Actinobacteria* that were initially completely dominant decreased to 44.2% after 5 months. After 12 months, *Cyanobacteria* (22.1%) appeared and remained dominant until thirty months. Thereafter, the population consisted of *Actinobacteria* (42.2%) and *Cyanobacteria* (57.8%). For the fungi, the *Ascomycota* phylum was dominant before treatment (100%), *Basidiomycota* (6.38%) appeared after five months, but disappeared after 12 months. After 30 months the fungal population started to stabilize and *Ascomycota* again dominated (83.33%). Green algae (*Chlorophyta*, Viridiplantae) were rare colonizers of the new material.

The results of this study indicated that final changes to the initial microbial populations were relatively small.

For many years, the only tests carried out on bioconsolidated stone were physical and chemical examinations, which gave information about the strength and chemical structure of the new material. There was no definitive knowledge about the ability of biocalcite to withstand the biological attacks to which it would be subjected in the environment. Indeed, Jroundi et al. [72] state that "It is unknown whether such a treatment is effective and does not produce any deleterious side effects under extreme hot and humid environmental conditions typical for the Maya area where . . . the potential for microbial biodeterioration is very high". In 2015, however, Shirakawa et al. [95] subjected fiber cement panels, treated in various ways to produce a surface layer of biocalcite, to biodeterioration testing in the hot and humid climate of Sao Paulo, Brazil. They found that the calcite formed by treatment with living *Bacillus sphaericus* LMG 222 57 plus urea plus B4 medium was the most resistant to the aesthetic biodeterioration that occurred within the 22 months of exposure. This resistance was correlated with lower water absorption and porosity, together with surface hydrophilicity, all linked to the smaller size of the biocalcite crystals. There was no apparent degradation of the crystals within the timescale of the test. Though promising, longer term experiments, often spanning several years, are necessary to truly know the extent of resistance of biocalcite to microbial attack under various environmental conditions.

4. Other Microbial Applications in Stone Conservation

Novel bioactive molecules based on microbial cells and products have also been used in biocleaning and protection of surfaces from microbial colonization, as discussed in the following sections. These biological techniques can be less invasive, and thus more acceptable, procedures than the traditional ones that use chemical treatments.

5. Biocleaning

Cleaning is one of the most important steps in the restoration process and normally comes before any other. When the building has special features, such as mural paintings, careful successive steps are necessary to remove the deposited polluting layers without affecting the original surface. The chemical and physical procedures traditionally used may have adverse effects on both the materials and the health of the restorer [96]. The recently introduced biological cleaning techniques that have been used on cultural heritage buildings in Italy, Spain and Greece, for example [97], avoid these problems. They may involve living bacterial cells or their hydrolytic enzymes. Both inorganic and organic materials can be removed gently from surfaces using these microbial products at temperatures that do not affect the underlying material.

5.1. Removal of Inorganic Materials—Black Crusts, Nitrate Crusts

Although some black crusts may be layers of dark pigmented cyanobacteria [3], those most commonly recognized on historic stone buildings are composed of gypsum (calcium sulphate dihydrate) and dark particulates from air pollution [98]. Traditionally, these are removed chemically with an ammonium carbonate-EDTA mixture, which may damage the underlying stone if not carefully applied. In 1992, Gauri et al. [99] reported a method for removing these layers from marble using a culture of *Desulfovibrio desulfuricans*, an anaerobic sulfate-reducing species better known for its ability to produce microbially-influenced corrosion of steel structures [100], and references therein). After various efforts to improve the treatment (e.g., [101,102]), it was shown to be effective in removal of the crusts and preservation of the noble patina beneath them and has now been applied successfully to various monuments (e.g., [103,104]). Care must be taken, however, with the duration of the treatment. Ranalli et al. [105] used sulfate-reducing bacteria to remove sulfate crusts and found that prolonged contact between the bacteria and the stone resulted in damaging sulfide precipitation.

Nitrate crusts are one of the types of stone efflorescences which are often considered to be of particular importance in deterioration of indoor artworks, such as mural paintings. Ranalli et al. [106] were among the first to suggest the use of nitrate reducing bacteria (a strain of *Pseudomonas stutzeri* in this case) to remove nitrate salts from stone. There have now been several successful cases of biocleaning of nitrates from stone; the wall paintings in the central vault of Santo Juanes church in Valentia, Spain, and the external wall of Matera cathedral are examples [107]. The latter building was treated with a novel carrier system (Carbogel) containing *Pseudomonas pseudoalcaligenes* that was covered with a PET film. Another novel application system was used on the mural paintings in the Santo Juanes church in Valentia [108]; *Pseudomonas stutzeri* DSMZ 5190 in a Japanese paper and agar layer was heated with infrared to a suitable temperature for optimum bacterial activity. Recently, Romano et al. [109] suggested that extremophilic bacteria might be good candidates for biological removal of efflorescences. Extremophiles live under extreme conditions, very high or very low temperatures, pH values and pressures, or in the presence of normally toxic agents such as irradiation and heavy metals. Often, they belong to the Archaea domain, but they can also be members of the Bacteria or the Eukarya (fungi or algae). The unusual conditions required for their growth means that their application as biocleaning agents should be safe, with no undesirable future effects on the environment or adverse effects on current lifeforms (including humans). This could make them more acceptable for use by conservators and restorers. Romano et al. [109] screened various extremophiles from the culture collection held by CNR, Italy, and selected an aerobic bacterium, *Halomonas campaniensis* 5AGT, as a potential remediation treatment for nitrate crusts. The bacterium lives at high pH values and is non-pathogenic.

Mixed salt crusts also occur and these have been removed by combined biological and chemical treatments ([110], and references therein). These crusts may contain not only sulfates and nitrates, but also carbonates, apatite and protein, hence posing a challenge to traditional techniques that could be overcome by these innovative methods.

5.2. Removal of Organic Materials

Heterotrophic microorganisms (those that utilize organic substrates) are very versatile, producing a wide range of enzymes that can break down many organic materials. Treatment with enzymes such as proteases and collagenases is already used by restorers to remove organic residues from paintings, for example, without affecting the base material [111]. Although microorganisms can be the source of such specific enzymes, it can be seen that the whole cells, containing a mixture of degrading enzymes, might be a more effective treatment in some cases, as well as being more economical. They can also be effective for otherwise difficult materials, such as casein, egg yolk, oil, and animal fat [110]. For example, Ranalli et al. [112] used cells of the bacterium *Pseudomonas stutzeri*, plus a final enzyme treatment, to remove organic residues, including animal glue residues, from wall paintings. The cost of the biological cleaning was much lower than that of conventional methods. More recently, the same group produced a new, improved, system, with an innovative carrier for *P. stutzeri* cells, for cleaning historic wall paintings [113]. Surface contamination of culturally important buildings by traffic fumes may also be susceptible to removal by microbial cells and their enzymes [114].

Rather than using whole bacterial cells, specific degrading enzymes can be isolated from them and used where the polluting material(s) is known. The addition of these enzymes to traditional cleaning methods can also enhance the results. Jeszeová et al. [115] successfully used a mixed enzyme preparation from the bacterium *Exiguobacterium undae*, containing proteases, peptidases, nucleases, peptide ABC transporter substrate-binding proteins and a phosphatase, to remove animal glue from 3 different types of substrate, glass, stone and wood from a range of trees species (*Quercus* sp., *Fagus sylvatica*, and *Picea abies*).

Microorganisms may be used to remove graffiti paint from heritage buildings. The modified alkyd binders and other organic polymers in the paints [116] are susceptible to some polymerases and lipases in microbial cells [116]. Sanmartin et al. [117] isolated a

number of paint degrading strains from a wide variety of environmental sources. The most promising bacteria belonged to the genera *Arthrobacter*, *Bacillus*, *Gordonia*, *Microbacterium*, *Pantoea* and *Pseudomonas*, while fungi of the genus *Alternaria* also proved interesting. In 2019, the same group tested the system on stone surfaces, using spray-painted granite and concrete coupons as the test surfaces [118]. The bacteria were grown prior to use in a culture medium enriched with powdered graffiti, to encourage the adaptation of the cells to the target paint. Macroscopic and microscopic examination, together with color and infra-red evaluations, indicated the success of the treatment. Germinario et al. [119,120] examined the effectiveness of lipase enzymes extracted from various bacteria and fungi for removing blue, green, red and black felt tip marker acrylic ink from unglazed ceramic substrates. An oil-in-water emulsion alone could remove the ink, but the addition of lipase increased the efficiency. The more gentle treatment with cells or enzymes, rather than more abrasive chemical removal, is an obvious advantage for use on culturally important monuments.

Microorganisms have also been suggested for removing biofilms, which contain high levels of protein, from historic buildings [121]. Biofilms are produced by microorganisms attaching to and thriving on the stone surface, and contain, besides the cells themselves, their metabolic products, especially EPS, and compounds that may be released by their lysis, such as proteins, lipids and nucleic acids [122]. In their mildest form, biofilms simply disfigure the surface, presenting a discolo red or dirty appearance; nevertheless, this requires treatment and, if possible prevention.

Valentini et al. [123] used the fungal enzyme glucose oxidase to clean the disfiguring patina from the surface of travertine and peperino stone from the Villa Torlonia in Rome. The hydrogen peroxide formed by the enzyme action was able to remove the thin biofilms, containing algae, cyanobacteria and heterotrophic bacteria, without etching the underlying substratum. The biocleaning method was especially recommended for travertine, whose somewhat lower porosity made it more resistant to peroxide etching. This biocleaning method was shown to be more efficient than the more traditional treatments using saturated $(NH_4)_2CO_3$ solution and EDTA in buffer and use of lipase enzyme.

Microbial cells have also been suggested as a control measure against biofilm formation. This relies on the fact that certain species can inhibit the growth of others, either by simple growth competition or by the production of antibiotic-like substances or other antagonistic metabolites [112,124,125]. This "biological control" is already used successfully in agriculture against plant pathogens (for example, [126,127]). However, the organisms that cause plant diseases are specific pathogens that can be inhibited by other (specific) organisms; cells growing in a biofilm on a stone surface will, unless the circumstances are unusual, consist of several species and genera. It has been suggested that antimicrobial substances produced by members of the genus *Bacillus* could be of interest in protecting stone surfaces from colonization ([110], and references therein), since this bacterial genus can produce a range of antibiotic substances affecting many types of bacteria; however, much work will be necessary before this becomes a truly viable option.

6. Conclusions

The great advantage of biorestoration based on microbial cells or their products, compared with traditional chemical, physical and mechanical methods, is that biological treatments are not destructive of the underlying substrate, simply removing unwanted overlying materials, in the case of biocleaning, or producing new stone, in the case of biocalcification. Biologically-based restoration also has the advantage of being recognized and appreciated by the community of restorers which are often reluctant to use synthetic, man-made materials. This is partially explained by the fact that ancient conservation practices are based on the use of biological materials as consolidants. Analogous behavior has been observed among other communities such as agriculture producers who are often keen to test biological-based methods in control of pests, but reluctant to use synthetic pesticides. The current COVID-19 pandemic has spurred interest in several sectors towards an increased use of green technologies.

Cultural heritage microbiology has relied heavily on fundamental studies at laboratory scale that do not reflect conditions that occur in historic monuments, where processes operate at the monument or archaeological site level, which encompasses a heterogeneous and complex set of conditions that hamper the assessment of microbially-based approaches for conservation. There needs to be convergence of cultural heritage with civil engineering, microbiology and biotechnology to encompass a longer timeframe and greater spatial scale that includes the building/archaeological site levels. Indeed, the best approach to stone conservation is an interdisciplinary one combining engineering, art conservation and biological sciences, including microbiology, biotechnology and environmental sciences [128]. Each discipline brings its own scientific and methodological culture to develop a holistic approach.

Biotechnology has emerged as a field perceived to be a crucial component in the knowledge economy, with potential in many fields, including conservation. Using novel microbial strategies need not only consider technical aspects of performance but also safety for human use, economics and skills. Transdisciplinary approaches are key to advance science and technology in cultural heritage conservation, but they can be challenging given the gaps in knowledge. Few restorers are trained in microbiology and biotechnology skills, but also few biotechnologists are trained in social disciplines such as anthropology and conservation science. For restorers, microbiology and biotechnology skills are key for isolation and assessment of performance of novel biocalcifying organisms and the optimization of culture conditions to cost-effectively produce microbial metabolites such as EPS. EPS with the correct chemistry, which is heavily controlled by cultivation conditions, is fundamental to control and further optimize the bioprecipitation processes, offering EPS and potentially other biopolymers as tools for biotechnological applications. If correctly chosen, biological methods are completely safe, not relying on toxic or potentially poisonous solvents. In addition, it has been shown that these methods can be more economical than chemical treatments. However, as with other biotechnologies, biotechnology for cultural heritage conservation needs to further develop its methods, confirm their reproducibility and show its economic benefits. Process innovation which relies exclusively on improving environmental performance (for environmentally-conscious conservators) does not give enough incentives, in particular for the private sector. Thus, a promising cost-benefit analysis at the economic, environmental and human health levels are necessary before a phase of consolidation and progressive acceptance by the concerned communities, including conservators, governmental agencies and the private sector, are recognized.

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