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Editor

Microorganisms in the Deterioration and Preservation of Cultural Heritage

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Chapter 11
Sustainable Restoration Through Biotechnological Processes: A Proof of Concept

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Abstract An understanding of the different microbial constellations or microbiomes, which every habitat and every organism harbor, will be the key to addressing many of the challenges humanity will face in the twenty-first century. Such comprehension could launch several innovations relating to natural and cultural capital, including historical and artistic heritage. In relation to cultural heritage, microorganisms are mainly known through their role as deteriogens, but the features creating damage can be exploited positively, attaining more sustainable restoration strategies, in accordance with the principles of compatibility and retreatability deriving from reflections on the Cultural Heritage inspired by the Charter of Venice (International charter for the Conservation and restoration of monument and sites (the Venice Chart 1964). In: ICOMOS, IInd International Congress of Architects and Technicians of Historic Monuments, 1964) onwards. In this article, we show a series of case studies, using both wild-type microorganisms and plant-based extracts, providing a comprehensive proof of concept of the feasibility of biotechnological solutions for a more sustainable restoration strategy, to replace the products in use which are often dangerous for operators, aggressive for works of art and no longer compatible with the environment. The overview of the case studies presented, many of which are still unpublished, responds to the need to go beyond the state of the art and has entirely sprung from suggestions by restorers, interested in learning about potential innovations and strongly determined to introduce non-toxic products in their daily work. In this perspective, the case studies dealt with two topics: bio-cleaning and disinfection. Noteworthy results were obtained on a platform of different types of artworks and different materials with compatible, harmless and selective products.

Keywords Bio-cleaning · Disinfection · Bio-restoration · Microbial biotechnology

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1 Introduction

The transition to products and practices that take environmental, social and economic aspects into account is today the challenge to be faced in order to develop a more sustainable approach to Restoration and Conservation, in accordance to European policies and the Strategic Innovation and Research Agenda (SIRA) of the Bio-Based Industry Consortium (2017). This represents a great challenge, not only for restorers and art historians, but also for researchers, politicians, scientists, entrepreneurs and also market players. Some of the products in use, recognized as harmful, are being eliminated from the market and others will be. Their replacement or reduction creates a space for new, more sustainable products in the market, with the possibility of creating a new space in the Bioeconomy chain.

Microbial biotechnologies play a full role in this process, thanks to their potential to provide new products with some advantages over traditional chemical-physical methods: selectivity thus low aggressiveness for the artifact, environmental compatibility, less danger for the operators, absence of ethical problems. The possibility of using bio-based techniques is perhaps a feasible option for the future, also in consideration of the attention that must be paid to the life cycle of a product. Microorganisms - and their derivatives - and products of plant origin are ideal candidates for the development of new bio-based products of renewable origin.

To date, however, despite numerous researches published in specialized journals, very few patents exist and very few products are available on the market. Especially, products based on microorganisms are currently absent in restoration market. The challenge must therefore point towards the transformation of research products into market products, available for restorers and ready for use.

To this end, it was necessary to broadly verify the actual potential of biotechnology, given the size and diversity of the objects involved, the enormous variety of materials used and the peculiar characteristics of the individual cases.

The case studies review described below (Tables 11.1 and 11.2) was planned by the authors to proceed beyond the state of the art (Ranalli et al. 2005; Bosch Roig et al. 2013; Gioventù et al. 2011).

The range of materials and the variety of cases involved proved that biological way is a good alternative to different products in use. Tailored solutions have been defined both to clean multiple coherent deposits and to control biodeteriogens using bacteria and plant extracts. The restorers, in order to find solutions to problems unsolved by the available methods, or to find effective alternatives to the toxic products in use, have submitted the case studies shown.

Table 11.1 Proof of concept for bio-cleaning by using bacterial strains

Artwork material	Deposit	Place (case number)	Bio-product	Outcome (reference)
Mural paintings	Overlayed layers of gypsum, carbonate, aged protein	Casina Farnese, Rome (1)	UI3, UT30, TPBF11	Patent ENEA WO2015040647[a]
	Shellac	Lab specimens (2)	CONC11, CONC18, LAM21	Preliminary trials[b]
Frescoes	Vinyl glues	Lab specimens (3)	FCONT	Preliminary trials[c]
	Primal resin	Galleria Carracci - Palazzo Farnese, Rome (4)	ZCONT	Positve (to be confirmed)
	Lime layer	Chiesa di San Giorgio, Bitti, Nuoro (5)	CER20, TPBF11	Negative, more research needed
Marble statues	Wax hydrocarbons	La lupa, Testa di Donna, Idealità e materialismo, Cleopatra- GAM, Rome (6)	LAM23, LAM30, OSS42, LAM21, SH7	Applicable[d]
	Carbonates, wax, Paraloid	Bacco col. cesto- La Venaria Reale, Turin (7)	ZCONT, TPBF11, LAM21	Positive for Paraloid[e]
	Iron oxides, copper oxides	Vatican Gardens (8)	FeIIC1, SME3.14	Positive (to be confirmed)
	Iron oxides	Lab specimens (9)	FeIIC1	Positive (to be confirmed)
	Deposits of unknown composition	Group of Michelangelo statues, Medici Chapels, Florence (10)	CONC11, ZCONT, SH7	Positive
Marble portals	Iron oxides, brownish spots, lab specimens	Galleria Carracci - Palazzo Farnese, Rome (11)	FeIIC1	Positive (to be confirmed)
Easel wood paintings	*Colletta* in oil	Madonna della Cintola, Vatican Museums (12)	SH7	Positive (to be optimized)[f]
Ancient paper	Animal glues	Archive document XVIII century (13)	TSRNS15	Applicable[g]
Calcography slabs	Zapon resin	Lab specimens (14)	LAM21	Preliminary trials
Stuccoes	Copper oxides	S. Prudenziana, Rome (15)	SME1.11	Positive

[a]Mazzoni et al. (2014)
[b]Grimaldi (2009)
[c]Cutraro (2014)
[d]Sprocati et al. (2017)
[e]Galizzi (2015)
[f]Crisci et al. (2020)
[g]Barbabietola et al. (2016)

Table 11.2 Proof of concept for biodeteriogens removal and control by using bio-products and bacterial strains

Artwork material	Deposit	Place	Bio-product	Outcome (reference)
Mobile painting on canvas	Biofilm	Private painting	Liq	Positive
Frescoes	Biofilm	Chapel XIII –Orta, Novara	Liq	Positive
Hypogeum	Biofilm	Domus Aurea, Rome	Liq,	Underway[a]
Stone material	Biofilm	Vatican gardens	Liq, BioZ, SME1.11	Positive
		Diocleziano termae, Rome	Liq, BioZ, SME1.11, NopalCap	Positive[b]
		Roman wall, Rome	Liq, BioZ, SME1.11, NopalCap	Underway
		Outdoor statues-Vicenza	Liq	Underway
Mortar, bio-mortar	Bio-receptivity	Lab specimens	Nopal	Positive[c]
	Biofilm	Outdoor statue "Beethoven"- Naples	NopalCap	Positive

[a]Rugnini et al. (2019)
[b]Ledda (2019)
[c]Persia et al. (2016)

2 Bio-Cleaning Procedures Developed in the Case Studies

The procedure involves the use of living bacteria immobilized at high concentration (10^8 CFU/mL) in support agents with different viscosities, to create a micro-pack, easy to apply and remove without leaving residues on the artifact.

The micro-packs are easy to apply and to remove, without dripping and leaving residues. They can be applied on vertical surfaces and ceilings; do not require strict operating conditions, in terms of temperature and pH and with no disposal problems. The micro-pack can be conveniently prepared on a support table or prepared directly on the object to be treated. In the first case a standard micro-pack is organized backwards: a plastic film, which preserves the humidity during the treatment, is first placed followed by a slightly moistened veil of paper (i.e. Japanese or English paper), a layer of bacteria in their support agent and another veil of paper. The interposition of the paper facilitates the application and the removal of the micro-pack and allows inspections to check the progress of the process. However, the opportunity to interpose paper can be evaluated from time to time, based on the specific situation. Direct contact with the original material can sometimes be more suitable. After removal, a damp swab or a sponge can easily clean the artefact.

The time of contact of the micro-pack depends on various factors, such as the nature of the substrate to be removed, the thickness of the layer; the metabolic rate of the microorganism, the nature and the state of conservation of the artifacts. The procedures developed in the case studies (Tables 11.1 and 11.2) are fairly

standardized, with overnight compresses, but they can be designed according to specific needs of the case.

Competent bacteria are selected according to the ability to metabolize the deposits to be removed, without damaging the original material of the artifact. For instance, to clean paper (case 13), bacteria must not show cellulolytic activity as well as to clean limestone they must not have the ability to solubilise carbonates. In this way the cleaning is selective and safe for the artifact.

The bacteria used in the case studies belong to the laboratory in-house collection ENEA (De Vero et al. 2019). The collection gathers over 800 strains of biotechnological interest mainly isolated from harsh environments, hypogea, artworks, etc. All strains are wild type and non-pathogenic (mainly belonging to the risk group 1), their gene sequences (rDNA16S) are deposited in the GenBank database.

The support agent must be compatible with the survival of the cells, without, however, representing a source for growth. The support material is decided on the basis of the needs of the surfaces to be treated and further properties, like as the ability to retain humidity and be malleable, absorbent capacity, total inertia, etc. Often the right combination between bacteria and the support agent determines the effectiveness of the treatment. Moreover, the suspension medium to embed bacteria influences the rheological characteristics of the support agent itself and the performance of the micro-pack. The suspension medium depends on the metabolic action that bacteria have to play for the deposit removal.

Organic deposits are degraded and used by bacteria as a carbon source for growth (biodegradation), while inorganic deposits (i.e. carbonates, phosphates, oxalates, oxides, etc.) are removed through solubilisation, mediated by secondary metabolites, such as organic acids and specific molecules. To best perform specific functions, the suspension medium must comply with the specific physiological needs of the bacteria.

In the case of the removal of organic deposits, bacteria are generally separated from the culture broth and re-suspended in a mineral solution to strengthen the attack of the target deposit. Sometimes cells are starved to deplete the internal carbon reserve in order to make the attack more effective. In other cases, for instance, when it is necessary to induce the production of enzymes (i.e. lipase, protease, cellulase, etc.) the bacteria must be left in their growth media.

When cleaning inorganic deposits, after separation from the growth media, the bacteria must be re-suspended in a solution containing a very low concentration of a carbon source in order to sustain bacterial metabolism for the production of metabolites (i.e. siderophores, organic acids, etc.) responsible for the solubilisation.

In the cases listed in Table 11.1, two products were mainly selected as support agents: Laponite®RD and Vanzan®NF.

Laponite®RD (BYK-Chemie GmbH, Germany) is a colloidal clay consisting of a mixture of silicates of sodium, magnesium and lithium. In the restoration, Laponite®RD is used to remove rust stains and ferrous encrustation on frescoes. It is sometimes used in combination with citric acid and sodium citrate, but also often with tap water, to avoid further damaging to already very compromised artifacts. It must be considered that Laponite®RD can dry off and shrink in some case.

Vanzan®NF (Vanderbilt Minerals LLC, Norwolk, CT) is the commercial name of the Xanthan gum, a high molecular weight exocellular polysaccharide derived from the bacterium *Xanthomonas campestris*. Xanthan gum is widely used as a rheology control agent for aqueous systems. It increases viscosity, helps to stabilize emulsions and prevents the settling of solids in a wide variety of applications.

Vanzan®NF shows a great compatibility with cells viability, perfect adherence and plasticity also on vertical surfaces, and overall the capacity to maintain the moisture without drying off or shrinking, up to 24 h of application.

Laponite®RD and Vanzan®NF were used for the first time as support agent for microorganisms in the bio-cleaning case 1 and 12, respectively (Mazzoni et al. 2014; Crisci et al. 2020). The most appropriate concentration for a correct consistency and property of the micro-pack was 9% w/v for Laponite®RD and 6% w/v for Vanzan®NF.

3 Bio-Cleaning Case Studies: Review and Discussion

The series of case studies (Table 11.1) springs from the European patent EP 3046779 extended to Italy, Switzerland, Germany, Spain, France, GB (Sprocati et al. 2014) that derived from the bio-cleaning of mural painting of the external loggias in the Casina Farnese (case 1). The cases that followed (2–15) expressly ranged between different types of artifacts, materials and substrates to be removed, experimenting numerous microbial species available in the in-house collection in order to create a proof of concept of the feasibility of microbial biotechnology to contribute to a more sustainable restoration strategy.

The Casina Farnese (case 1) is situated at the top of the Palatine Hill, in Rome, the double order of open loggias representing the legend of Ercole and Caco was completed by 1593. The decoration of the lower loggia, object of the bio-cleaning tests, was ascribed to a pupil of Taddeo Zuccari and represents Ercole killing Caco. Coherent deposits composed of gypsum, weddellite, calcium carbonate, apatite, nitrate, and aged proteinaceous matter (FTIR-ATR analysis) were deemed difficult to remove from the wall paintings, especially in areas where the deposits were superimposed in layers. Following a laboratory screening, three bacterial non spore-forming strains were selected to solubilise calcium sulphate and carbonate, to degrade protein, to solubilise inorganic compounds and degrade protein material. Micro-packs with single strains were used in situ, from July 2012 to February 2013, in temperatures ranging from 6 °C to 37 °C. After experiencing some supports in use for wet compresses without satisfactory results, the living bacterial cells were eventually immobilized in a Laponite®RD gel. In this occasion, Laponite®RD was used for the first time to immobilize microbial cells, proving to be optimal both for compatibility with the vitality of the cells and for perfectly retaining the material of compress, without leaks. The strain *Cellulosimicrobium cellulans* TBF11^{E} DSM 27224 (GenBank accession number: EU249577) removed the inorganic darker layer, *Stenotrophomonas maltophilia* UI3^{E} DSM 27225 (JX133197) dissolved the

Fig. 11.1 Aged-casein layer covering the architrave over the door at Casina Farnese, Rome. (**a**) before cleaning, (**b**) after bio-cleaning with a micro-pack containing UI3 strain, (**c**) after the pictorial retouching (photo from Mazzoni et al. 2014)

brownish layer (probably aged casein) and *Pseudomonas koreensis* UT30^{E} DSM 27226 (JX133187) removed the mixed deposits. In areas with overlaid deposits, micro-packs were applied in succession, containing the strain able to remove each layer.

Another critical point threating the survival and the performance of bacteria was the low temperature in the open loggia on winter days. No significant differences were detected in the efficacy of the bio-cleaning treatment, probably the micro-pack contributes to maintain a microclimate suitable for the bacterial metabolic activity. After the bio-cleaning, the restorers successfully completed the restoration (Fig. 11.1). The microbial monitoring performed on treated surfaces up to 4 months detected only transitory population, but none of the used bacterial strains.

The procedure established by this study proved to be selective, safe for the mural painting, uncaring of the seasonal temperature changes. As a result, the cleaning was gradual, controllable and able to safeguard the surface of the painting. Laponite®RD may have facilitated the cleaning process by softening the layer of deposit to be removed, as seen in Rozeik (2009), with a synergistic effect (Mazzoni et al. 2014).

This case faced three challenges: coherent overlaid deposits of different nature; choice of the right support, suitable for application on vertical surfaces and ceilings without releasing water and bacteria, and low temperatures. The identified procedure paved the way for the case studies that were addressed later.

A few cases are preliminary studies carried out only at laboratory scale on specimens reproducing the artwork. The cases 2 (shellac) and 3 (vinyl glues) deals with the removal of fixatives (natural and synthetic resins and adhesives based on proteins and hydrocarbons) from mural paintings and frescoes, used to repair the flaking or disintegration of the pigment layer; loss of cohesion or adhesion to the support.

Case 2 was focused on shellac degradation. The use of shellac as a fixative for mural paintings was very widespread in India being used for the restoration of paintings kept in the caves of Ajanta, Ellora, Bagh or Kancheepuram (Sharma et al. 1980; Singh and Balasaheb 2013). Although shellac shows good penetration features, adhesive properties and resistance to biological attacks, the resin has a tendency to yellowing and darkening, becoming hard, brittle and insoluble, because of polyesterification reactions which are accelerated by high temperatures. This

makes shellac one among the undesired deposits which can be the object of cleaning, necessary to re-establish the visual perception of the paintings (Mora et al. 1984).

In the last thirty years different cleaning procedures, which have involved the use of various solvents, have been carried out: starting from aqueous solutions of sodium or potassium borate, carbonate or hydroxide, to mixtures of different solvents, such as butyl lactate, morpholine, alcohol, turpentine oil and ligroin, applied in succession and for the final cleaning. In exceptional cases, in order to make the deteriorated and harden, superficial layer of shellac susceptible to the solvent attack, a solution of formic acid 1:10 in ethyl alcohol have been used. As drawbacks, colour loss, white crusts and surfacing of soluble salts appeared after each treatment. Case 2 represented the first attempt of removing shellac through a biological approach (Barbabietola 2013).

On the molecular level, shellac is a complex mixture made of mono-and poly-esters of hydroxyl-aliphatic and sesquiterpenoid acids (Colombini et al. 2003). Comparing fresh with naturally aged shellac analysis showed that the only stable compound under pyrolysis condition was the butolic acid that is used as a marker for the molecular pattern recognition of the molecule.

In order to carry out in vivo bio-cleaning trials, specimens of wall paintings were purposely prepared. The specimens were made up of bricks covered with a plaster layer. Three different pigments, cinnabar, (red), kaolin (white) and indigo (blue), were diluted into three different media, rabbit skin glue, arabic gum and linseed oil and then were spread by brush over the plaster layer. When the paint layer was completely dried, a solution of shellac in ethanol 10% (w/v) was spread by brush over the upper surface of the wall painting specimen. In order to simulate the climatic conditions of the Ajanta caves paintings, a climatic chamber was set at 34 °C and 96% R.H, which represents the annual average values of the Maharashtra region where Ajanta caves are situated. Colorimetric measurements with the spectrophotometer Techkon sp-820λ were carried out on the samples, before they were subjected to artificial weathering. In order to monitor shellac transformation as a result of the microbial growth, gas chromatography coupled with mass spectrometry was employed.

Among the many strains tested, only three, *Pseudomonas stutzeri* CONC11 (EU275358), *Achromobacter xylosoxidans* CONC 18 (EU275351) and *Acinetobacter calcoaceticus* LAM21 (JK534248) were able to grow on shellac 0.2% as sole carbon source. A new procedure to supply shellac on a paper disk was developed, in order to achieve reproducible chromatograms for gas chromatography-mass spectrometry analysis, aimed to determine which components of shellac were able to sustain the bacterial growth. Butolic acid was reduced by 60% after 24 h of incubation and by 80% in the plateau phase and seemed to be the only component that undergoes a transformation after microbial growth. Work is actually in progress in order to tailor a microbial formula by varying the successions of the bacterial strains over time, so that to strengthen the attack to more complex compounds, once assimilated the more soluble portion.

Case 3 deals with the removal of vinyl glues from the frescoes of the Chapels of Saint Martial and Saint Jean of the Pope's Palace in Avignon, the most important

Gothic palace in the West word, built between 1335 and 1364. The frescoes, realized by an Italian painter, Matteo Giovannetti, constitute an exceptional masterpiece. The conservation of these paintings as a whole is to be considered a miracle, given the many damages to the Pope's Palace over the centuries. In a previous restoration, in the '70s, vinyl glue was applied as consolidating agent and, following ageing, proved detrimental for a correct reading of the frescos.

The removal of the resin (Vinavil K40) with products in use was critical for restorers and previous works on the biological removal of Vinavil K40 were unknown in the literature. The research aimed to find microorganisms able to attack the resin. Research started from the isolation of microorganisms inhabiting the surfaces covered with the resin. The 42 bacterial strains isolated from the frescoes together with microbial strains of the in-house collection have been tested for the ability to oxidize vinyl glue and produce esterase enzymes. Few strains showed a marked esterase activity; the best performing was the fungal strain of *Penicillium commune*, FCONT. The removal tests were performed on fresco specimens reproducing the technique used by the painter Matteo Giovannetti. He made significant changes to the traditional fresco technique, by eliminating the laying of the first wall layer and giving an unusual importance to the lights with a dry laying on the composition to improve the shine. Specimens were covered with pigments (yellow ochre, red ochre, green earth and ultramarine blue), on half of the surface a layer of Vinavil K40 dissolved in about 70–80% w/v acetone was applied. Some of the specimens have been aged in a climatic chamber for 38 days under UV light, at a constant temperature of 25 °C and 45% R.H. Laponite®RD micro-packs containing microbial suspensions were applied with a succession of 2 applications for 24 h each one. The best results were obtained with *Penicillium commune* FCONT. The efficacy of the treatment was assessed using three diagnostic techniques: Laser Induced Fluorescence (LIF) and Attenuated Total Reflectance Infrared Spectroscopy (ATR) that recorded a marked reduction in the area of the peak of the Vinavil K40 after treatment with the fungal strain (∼50%). Electronic Scanning Microscopy (SEM) with Microanalysis (EDS) revealed a clear alteration of the vinyl film after the bio-cleaning treatment. The study led to define a biological process mediated by a fungal strain able to attack the vinyl glue. For bio-cleaning applications it would be better to favour the use of non-spore-forming strains. In this case-study, however, the strain is applied in the physiological state of hyphae and the contact time useful for attacking the Vinavil is less than the time required for sporulation (Cutraro 2014).

Primal resin was the problem faced by the restorers in the restoration of the frescoes in the Carracci Gallery at Palazzo Farnese, in Rome (case 4). Carracci Gallery is considered the triumph of Baroque mural painting. The frescoes were affected by widespread small/medium-sized stains, due to the darkening of the resin used in the '70s, as a consolidator of the numerous cracks that occurred following a landslide of Tiber river. The fungal strain *Penicillium commune* FCONT and the bacterial strain of *Rhodococcus* sp. ZCONT (KY697119), previously selected as candidate for the Vinavil bio-removal experiments, were tested. The strains were separately immobilized in micro-packs of Laponite®RD and applied for 24 h on the frescoes. The micro-packs containing *Rhodococcus* sp. ZCONT perfectly removed

the resin layer without damaging the different pigments present in the different areas of application.

Several of the case studies concerning stone material (case 6–11) have been carried out on the artifacts. Marble lends very well to this type of treatment with wet-packs applications, as there are generally no negative implications, despite those occurring in other situations, such as on wood or canvas paintings.

Some statues of the National Gallery of Modern and Contemporary Art, in Rome, have been treated with bio-cleaning (case 6). "La Lupa" by G. Graziosi, inspired by Rodin (Count Ugolino statue) represented an interesting and unusual case. La Lupa remained outdoors in the gallery gardens about 40 years and the main problem were large areas completely blackened by deposits of urban smog, penetrated over time in the marble matrix. These alterations were impossible to clean with traditional methods without negative consequences for the statue.

Given the nature of urban contaminants, a microbial formula consisting of three bacterial strains *Exiguobacterium* sp. LAM23 (EU019988), *Bacillus cereus* LAM30 (EU019990), *Bacillus subtilis* OSS42 (EU124568), degrading oil and growing on hydrocarbons as the only carbon source, have been used for bio-cleaning. The strains were isolated from the polluted site of Bagnoli and previously used for the bioremediation of diesel and heavy metals contaminated soil (Alisi et al. 2009; Sprocati et al. 2012). These bacteria produce extracellular surfactants through which they are able to solubilise hydrocarbons and make them available for biodegradation. Large areas of the statue were treated with the bacterial formula immobilized in Laponite®RD gel, by applying in succession three micro-packs for one night each one. The synergistic effect of bacteria producing bio-surfactants and Laponite®RD with absorbent power, has allowed acting in depth, leading to a significant lightening of the marble matrix. The combination of bio-cleaning with a traditional washing, performed to remove other common deposits, allowed to obtain a satisfactory degree of cleaning for the exposition.

Other statues of the Gallery, already treated with a traditional cleaning, still had residues of waxes and fats, not further removable with usual products. The sculptures "Testa di Donna" by E. Quadrelli (Fig. 11.2), "Idealità e Materialismo" by G. Monteverde and "Cleopatra" by A. Balzico have been successfully cleaned using different strains capable of removing waxes: *Acinetobacter calcoaceticus* LAM21, (JK534248), and fats and oils: *Serratia ficaria* SH7 (KP780180).

In collaboration with the Cultural Heritage Conservation and Restoration Centre "La Venaria Reale" of Turin, it has been performed a series of bio-cleaning tests on the "Bacchus with basket", a Roman statue in Greek marble, property of the Turin Museum of Antiquities (case 7). The restoration of the statue involved the reassembly of all the fragments separated in the early twentieth century and has foreseen, in the cleaning phase, the execution of experimental tests for the removal of various deposits, the nature of which had been diagnosed by the FT-IR and XRF analysis. The bio-cleaning has been tested on a piece on right leg, left dirty after cleaning with saturated ammonium carbonate. The FT-IR analysis identified the deposit as Paraloid B72. The appearance of the piece was a black/brown and removal tests with acetone had no effect.

Fig. 11.2 Testa di Donna- Quadrelli. The portion treated with a micro-pack containing living cells of LAM21 strain was cleaned in an overnight application

A single micro-pack containing the bacterial strain *Rhodococcus* sp. ZCONT immobilized in Vanzan®NF was applied overnight on the half of the dowel covered by Paraloid B72. After removal of the pack, a slight mechanical action with a diluted acetone-soaked swab was sufficient to completely remove the Paraloid residues, discovering the completely browned surface. FTIR spectra, before and after the treatment, confirmed the effectiveness of the treatment. Restorers reported that the noble patina of the original material was preserved unlike other more aggressive products (Galizzi 2015).

In the case of the pedestal of a statue in the Vatican gardens (case 8) copper oxides were responsible of stains. The patches have been treated with micro-packs containing the bacterial strain *Sphingopyxis macrogoltabida* SME 3.14 (MT023684) immobilized in Vanzan®NF. The strain was isolated from a mine in Sardinia and it has been selected for its capacity to produce siderophores, to have a high esterase activity and to not be able to solubilise carbonates.

The case 11 faces with brown stains on marble. During restoration of the Carracci Gallery (Palazzo Farnese, Rome), the sixteenth century portals of Carrara marble presented brown and other rust-coloured stains due to pins or brackets used for the repair of cracks due, as well as for the frescoes, to the past landslide of Tiber river. In this case the restorers put forward the hypothesis that the brown stains present on the sixteenth century portals of the Gallery may be due to an emergence of the iron contained in the marble, due to maintenance or cleaning treatments previously carried out, probably with acids and waxes.

Staining of the stone can be attributed to corrosion of inclusions of iron minerals present in the same stone or oxidation of metal parts used as elements purely aesthetic or for structural strength. The chemical removal of stains of iron compounds on marble shows the drawbacks encountered using phosphates (aggression of the limestone), oxalates (not very effective), fluorides (good solubility of iron in a saturated solution of ammonium fluoride), salicylates (unusable as it has a strong red colour). Alternatives could be phosphoric acid, phosphoric acid and EDTA disodium salt in water, glycerine and gypsum, reagents that reduce Fe^{3+} to Fe^{2+}, and then re-oxidize producing new iron hydroxides or hydrated salts more easily removable. It has experimented the use of another complexing agent, the thioglycolic acid neutralized with ammonia, which has produced good results but it is restricted in the use because of the toxicity for the operators. Even cysteine has been proposed to treat the iron stains on marble. It is an amino acid with a high structural similarity with thioglycolic acid, but not toxic, and it has an amino group, which gives it three complexing groups. Cysteine can act both as bidentate and tridentate binder, the latter in particular with Fe^{3+}through the thiol and carboxylic groups. Having three donor groups and being the reactions variable according to the pH and the redox potential, it has a chemical very complex, and its chemical and kinetic behaviour is very difficult to understand. All the reactions, however, give a dark colour to the end product, which can vanish with the time.

A biological approach is based on the exploitation of bacteria producing siderophores, water-soluble molecules with low molecular weight, which can bind in a specific manner Fe^{3+}. Bacteria have acquired the ability to synthesize and secrete siderophores to overcome the shortage of bioavailable iron in nature. Siderophores are usually released into the extracellular environment, where they act as a captor of iron. Specific cell receptors allow the entrance into the cell of the complex Fe-siderophore. Once in the cytoplasm of the cell, the complex dissociates, with reduction of ferric iron to ferrous iron. The siderophore empty of the reduced iron can be degraded or reused releasing it into the extracellular medium. This topic has been addressed in a preliminary work with a series of tests performed on laboratory specimens prepared ad hoc, reproducing rusts on different stone materials (Pieragostini 2015).

On the Carracci Gallery portals we experimented the bacterial strain *Pseudomonas protegens* FeIIC1 (KY765342), isolated from mine drainage waters and selected for its high siderophores production.

After preliminary test on small portions, Vanzan®NF micro-packs containing the bacterial strain FeIIC1, added with a very low concentration of cysteine (0.1% w/v), were applied overnight on almost the entire surface of the four portals. The rust disappeared and the brown stains inside the crystalline matrix had lost their brown colour or were largely lightened. After the treatment, a very slight dark nuance due to cysteine, vanished with the heat of the day without leaving a trace.

The most recent study on marble was carried out on Tombs of Giuliano and Lorenzo de' Medici sculptured by Michelangelo in the Medici Chapel in Florence (case 10). It was not available a precise diagnosis of the composition of the deposits to be cleaned, as merely non-invasive analyses were performed. Based on the

conservative history, restorers referred that casts have been made, the first by Vincenzo Danti around the '70s. Release agents for casts were presumably animal gelatine, oils and mixtures with soaps, used in bronzes at the time. The restoration that followed left some residues and presumably applied protective agents, such as waxes, which over time changed to a yellowish-orange tone. Withdrawals with swabs made in these areas did not give indications (personal communications by restorer M. Vincenti).

As for the conservative history of the sarcophagus of the monument to Lorenzo Duke of Urbino, the substantial traces of organic material should have been present since ancient times. The diffractometric analysis on the patina sample on the back of the sarcophagus revealed the presence of inorganic compounds (calcite, silicate, chalk). Infrared spectroscopy performed on the blackest sample suggests the presence of phosphates, which realistically could refer to the hypothesis of body fluids.

Combining historical reconstruction with the few analytical results, useful bacterial strains have been chosen from the in-house collection and tested. The marble rear floor of the altar of the chapel offered a palette to experiment with different bacterial strains for cleaning coherent generic dirty deposits. The bio-cleaning tests were performed directly on limited areas of the sculptures.

Eight different bacterial strains have been individually immobilized both in Laponite®RD and Vanzan®NF micro-packs. Depending on the specific characteristics of the portion to be treated, Laponite®RD and Vanzan®NF micro-packs were chosen. On statues, exclusively Vanzan®NF was used, for technical needs or for opportunity assessments. Vanzan®NF is a necessary choice when the surface to be treated is not smooth, such as in the case of locks of hair, ears, facial folds; moreover Vanzan®NF does not have absorbent power in itself, perfectly retains humidity and does not shrink.

Thus, on the head of Duke Giuliano a bio-cleaning was performed first using a micro-pack with Vanzan®NF without bacteria. On the head of the Night, good results have been obtained treating the hair with *Pseudomonas stutzeri* CONC11 and an earmuff with *Rhodococcus* sp. ZCONT, the deposit has been removed without interfering with the marble below.

On the sarcophagus and the altar floor, both Laponite®RD and Vanzan®NF micro-packs were tested for each strain, in order to assess which was the most effective combination between bacteria and support agent.

On the sarcophagus three strains have been tested: *Rhodococcus* sp. ZCONT with lipase activity, degradation resin, oil degradation, bio-surfactant production, calcite precipitation, no proteolytic activity, *Pseudomonas fluorescens* LAM33 very reactive for phosphates solubilisation, and *Cellulosimicrobium cellulans* TBF11, for carbonates solubilisation. The control micro-packs did not produce significant differences compared to the situation prior to treatment, nor between them. Among the tested strains, *Rhodococcus* sp. ZCONT was truly active on the coherent deposit and the best cleaning quality was obtained with the strain applied in Laponite®RD, providing a very good cleaning quality, for the degree of cleanliness, homogeneity and the respect of the proportions.

The back of the sarcophagus, where very dark, sometimes black stains were present and phosphates had been detected, was treated with the *Serratia ficaria* SH7 strain in Laponite®RD, obtaining a total removal of the deposit, except in the thickest points, where it was necessary to proceed with several applications.

By the literature, the artworks undergone bio-cleaning are mainly frescoes, mural paintings and stone (Bosch-Roig et al. 2014; Mazzoni et al. 2014). Case 12 faced a challenge, it concerned the removal of an aged darkened organic deposit from a fragile surface of a sixteenth century wood painting. The experiment was carried out in collaboration with the laboratory of paintings and the Scientific Research Cabinet of the Vatican Museums, where the painting was under restoration.

Main challenge was to meet the conflicting requirements of microorganisms - needing aqueous solutions- and painting - refractory to a long exposure to moisture. The final strategy was to couple the bio-cleaning with a chemical pre-treatment as protective of the fragile painted surfaces (Crisci et al. 2020).

The deposit consisted of an animal glue with oil, called "colletta con olio", very tenacious, altered and resistant to the usually effective chemical treatments without further damaging the painting. The original recipe includes different oils, gall and molasses.

Two original bacterial strains, *Serratia ficaria* SH7 and *Pseudomonas protegens* FeIIC1 non-pathogenic and no spore-forming, were selected following a metabolic screening, where different bacterial strains have been tested on the individual substrate composing the original recipe.

Artificial wood specimens, simulating the artistic technique and covered by a layer of "colletta con olio", were weathered and used as laboratory tests, to simulate the application on the artefact. After tests on the specimens, using different support agents and different application times (from 8 to 24 h), the best procedure resulted in applying the strains entrapped in Vanzan®NF, used for the first time to this purpose. Micro-packs allowed a complete removal of the "colletta" without altering the colours of pigments, nor raise the pictorial film and without causing swelling of the wood. The results allowed applying the procedure directly on the wood painting, where this effective combination led to the selective removal of the deposit, through a long-lasting contact of bacteria with the sensitive and hygroscopic material of the painting, without damage for the artwork.

This experiment was significant, as it demonstrated the possibility of degrading very tenacious and refractory deposits without the use of toxic products; the possibility of proceeding with the bio-cleaning method even on materials sensitive to humidity and on very fragile surfaces. In perspective, an optimisation of the procedure can be feasible by acting on microbial metabolism, to reduce the application time needed to a complete removal of tenacious deposits.

Animal glues were historically used both in paper manufacturing, during the sizing process, and in conservation as adhesive for the lining of prints or graphics and for the creation of *passe-partout.* Animal glue films are highly hygroscopic and go through degradation by ageing. The development of internal stresses affects the glue's elasticity, strength and physical stability and may lead to significant damage to the substrate. Humidity, temperature, UV radiation and pollutants can induce

deterioration phenomena, such as protein cross-linking, hydrolysis of peptic bonds, oxidation, while the presence of microorganisms can lead to the production of acid metabolites and pigmented spots, which cause a strong optical-chromatic alteration. So, the removal of glue staining and the detachment of prints from the aged support become an essential step in the restoration and conservation of paper.

The chemical and mechanical methods in use, show serious drawbacks, whiles do not offer appropriate solutions, are too aggressive and use toxic products. To date, the use of enzymes is the only bio-based method. The need for skilled operators, together with the optimal application conditions required (high temperature, stable pH conditions, favourable saline concentrations), and the high costs have created difficulties in mastering the enzyme use so far.

Following a screening, a bacterial strain *Ochrobactrum* sp. TSNRS 15 (EU249585), deriving from an Etruscan hypogeum, was selected from the laboratory collection, able to grow on Cervione and rabbit glues as sole carbon source and devoid of cellulolytic activity. Original paper samples, kindly supplied by the National Institute for Graphics (case 13), representing the back supports of ancient prints from a historical volume of prints from the collection of the Institute, have been treated with the bacterial strain immobilized in an agar gel. The treatment showed its efficacy already after 4 h of incubation, allowing the complete removal of the thick layer of glue from the surface of the paper specimen. The colorimetric measurements allowed to assess the whitening of the specimens, by the increase in the L* and the decrease in the b* coordinates. SEM observations showed cellulose fibres appearing from the compact paste layer after 4 h of treatment, demonstrating the disappearance of the adhesive layer consumed by bacteria as a carbon source. To avoid undesired secondary colonization due to the remains, a final check verified that no undesirable residues were left over after the treatment.

The removal of copper oxide stains from decorative stuccos in the chapel of San Pietro, in the basilica of Santa Prudenziana (case 15, Fig. 11.3), at Rome was tested through bio-cleaning with the bacterial strain *Sphingopyxis macrogoltabida* SME 3.14 in Vanzan®NF 6% W/V. SME 3.14 is a producer of siderophores and shows esterasic activity. The stuccoes had light, medium and high intensity green stains, due to the oxidation of the "porporine" made of brass-based alloys (copper, zinc,

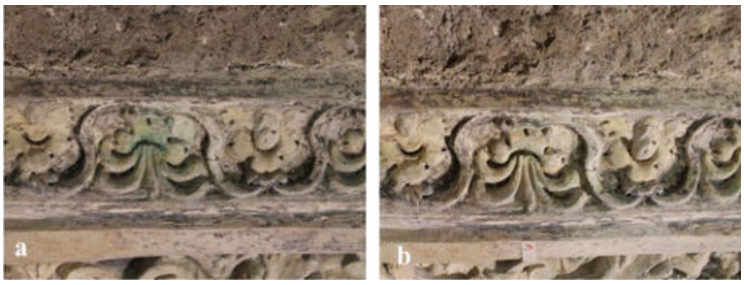

Fig. 11.3 S. Prudenziana stuccoes with copper oxides blue/green spots (**a**), removed after an overnight application with a micro-pack containing SME 3.14 strain (**b**)

iron) used in previous restoration works to replace the original gilding. Nine points with different intensities were treated. The bio-cleaning tests were carried out both by applying the micro-packs by direct contact and by interposing a tissue paper. Each application was in contact for 24 h. The most intense spots were cleaned with two consecutive applications. Dino-Lite digital micro-images taken for each point before and after the treatment demonstrated the fading or an attenuation of the stains.

The application of Vanzan®NF micro-pack in direct contact with the surface of the stucco, without the interposition of paper, led to a greater adhesion of the supports to the stucco, with greater risk that the product could not be completely removed; in fact, in the portions where the product was left for 48 h the stucco absorbed part of the water of the support, making it considerably more adherent to the substrate.

As far as efficacy is concerned, we do not yet have enough data to establish which application method is preferable (by placing an interface or directly in contact) or which application time can be recommended.

Cleaning of stucco from copper oxides is therefore biologically viable, but the procedure requires further tests to be optimized.

4 Biodeteriogens Removal and Control

The term biodeterioration was defined about 50 years ago by Hueck (1965) as "any unwanted change in the properties of a material, caused by the vital activity of living organisms". In many natural environments, the physical and chemical transformations of materials are considered necessary and positive conditions; however, on artistic material the transformations of the substrate induced by the colonizing microflora, in association with various environmental factors, are seen as a destructive and negative phenomenon, both from a cultural and economic point of view. For a long time, abiotic factors were thought to affect physicochemical properties, facilitating subsequent microbial colonization. Furthermore, it was believed that the phenomena of biodeterioration would modify the substrate only from an aesthetic point of view, that is, inducing the appearance of patinas and coloured spots on the surface, following the release of biogenic pigments. Recently, several analytical approaches have demonstrated that microorganisms are also responsible for physical, chemical and aesthetic modifications of the colonized surface (Sterflinger and Piñar 2013). They can use the surface as growth support, using mineral components or surface deposits as metabolites necessary for their development. The speed and extent of microbial colonization are influenced not only by environmental parameters, such as the availability of water, but also by the physical-chemical properties of the material, such as the mineralogical composition. In fact, although microorganisms colonize a certain environment permanently, their harmful activity is not constant, but periodic.

Today the concept of biodeterioration is intended with a broader meaning: it is known that the phenomenon is controlled by synergistic and antagonistic

relationships between colonizing microflora and environmental factors; therefore, it cannot be considered as an isolated phenomenon, as it occurs in conjunction with other phenomena of physical and chemical degradation, and it is therefore difficult to attribute any specific damage to a single cause.

Currently, to control or eliminate the phenomena of biodeterioration, restorers can intervene with mechanical (chisels, spatulas, scalpels), physical (UV rays) and chemical methods (biocides).

The biocidal products have been classified by the EU in four categories and twenty-two product-types and their use is regulated under the Biocidal Products Regulation (BPR, Regulation (EU) 528/2012, Biocidal Products Regulation (EU) 2012) (replacing the Biocidal Products Directive 98/8/EC 1998). Nowadays, many of the broad-spectrum biocides have severe limitations in their use for the toxicity and persistence in the environment. In conservation and restoration, only few of the existing biocides can be used, due to the lack of information about their interaction with the historic materials of the artworks.

Benzalkonium chloride, one of the most used substances in disinfection as part of the formulation of many commercial biocides used by restorers is being reviewed for use as a biocide in the EEA and/or Switzerland according to the ECHA site (2020).

Another aspect not to be underestimated in the use of biocides in the restoration is the onset of resistant microbial strains. As it happens in clinical microbiology, where the thoughtless use of antibiotics determines the development of resistant microorganisms, the repeated use of the same biocide or the choice of incorrect concentrations can determine the selection, in the microflora present on the artwork, of strains resistant to the biocide used. This phenomenon is very serious because it can determine the onset of attacks by biodeteriogens that become difficult to eradicate, considering that often the resistance acquired extends to the whole class of biocides to which the product used belongs. This was exemplified in Lascaux Cave, France, where the indiscriminate use of benzalkonium chloride resulted in an explosive bacterial and fungal attack (Bastian et al. 2009; Martin-Sanchez et al. 2012).

4.1 "Green Biocides"

There is an increasing interest in the use of naturally-produced compounds, and the need of alternative solutions led the researchers to explore the world of the natural substances such as plant extracts or bio-based products, that will be more easily degraded and environment friendly. Plants extracts are already used as biocides in food, medicine and different fields of the pharmaceutical industry. In the last years they have been tested for other applications, such as in the biodeterioration control of Cultural Heritage as a valid alternative to the traditional biocides (Rotolo et al. 2016). Many of these compounds are phenols, polyphenols, terpenoids, essential oils, alkaloids, lectins and mixtures of polypeptides derived from plants (Guiamet et al. 2006).

Oregano, thyme, clove and arborvitae essential oil have been tested for assessing the antimicrobial potential and might be used as broad-spectrum antimicrobial agents for decontaminating an indoor environment (Puškárová et al. 2017). A pilot study was carried out at the Vatican Gardens (Devreux et al. 2015), the applications of essential oil of thyme and oregano have been compared with chemical products based on quaternary ammonium salts such as benzalkonium chloride, on stones affected by biodegradation. The first results obtained showed that the synergistic use of the two essential oils allows to obtain the best biocidal efficacy. Recently an anti-musk product based on essential oils from plant became available on the market (Essenzio, Ibix Biocare). Anyway, essential oils use is not free of concern, since lavender derivatives, including essential oil, are listed in REACH registrations among the substances that cause "serious eye irritation, are harmful to aquatic life with long-lasting effects and may cause an allergic skin reaction".

Many bio-based substances have been investigated till now, as reported in a recent extensive review (Fidanza and Caneva 2019) that lists 61 natural substances, among essential oils and substances of plant and microorganism origin. Their application produces highly variable results due to a lack of a coherent assessment of the best practices, showing the need of a standard methodology in the use of natural biocides for controlling biodeterioration.

For these reasons, our effort was to develop some alternative ways to remove the biological patinas and possibly control the further microbial development, all of them based on natural products of plant and bacterial origin that are harmless for the health and the environment, using a standard procedure involving the pack application. The bio-products have been applied in the real-case studies listed in Table 11.2; here we describe briefly their origin, characteristics and range of applications performed until now.

4.2 BioZ

Surface active agents or "surfactants", are a group of molecules that lower the surface tension between two liquids or between a liquid and a solid. In the practical sense, surfactants may act as wetting agents, emulsifiers, foaming agents and dispersants. Bio-surfactants are synthetized by microorganisms and they are being studied for antimicrobial properties and their potential use as biocides on artefacts (Grimaldi 2009; Rivardo et al. 2009; Desai and Banat 1997). Their action is still unclear; they may inhibit infections through signals that interact with the host and/or bacterial cells or prevent the microbial adhesion and growth on various substrates (Rodrigues et al. 2006). Some bio-surfactants may show antimicrobial activities which could be applied to conservation of cultural heritage.

BioZ is a crude extract containing extracellular glycoproteins, produced by the strain MCC-Z (JF279930) from the ENEA-Lilith collection. The strain was identified by 16SRNA sequencing and belongs to the *Sphingobacteriaceae* family and *Pedobacter* genus.

The product BioZ has a good surfactant and emulsifying action which is preserved after freeze-drying: reduces the surface tension by 40 mN/m and generates an emulsion stable over time, temperature, pH and salinity, with almost constant values up to 4 months, ensuring the effectiveness in multiple application conditions (Beltrani et al. 2015).

BioZ product does not contain live bacterial cells, since its preparation involves an autoclave treatment at 121 °C for 20 min, it is applied incorporated in a suitable support to the specific situation to facilitate its application and removal without leaving residues on the surface.

The emulsifying capacity of BioZ has been tested on many hydrocarbon compounds (hexadecane, toluene, xylene, isooctane, cyclohexane and diesel) which represent common environmental contaminants and are often included in the superficial deposits of artistic artefacts. BioZ could also facilitate the removal of unwanted deposits of dust and smog particles, which often cover the surface of artifacts exposed to atmospheric agents. Its emulsifying activity was tested in the cleaning of laboratory samples from fumes of fuel oil, fats and kerosene.

BioZ antimicrobial activity was tested in-vitro on microorganisms known to be responsible for biodeterioration of Cultural Heritage, such as *Cellulomonas* sp., *Bacillus cereus*, *Bacillus pumilus*, *Bacillus megaterium*, *Acinetobacter calcoaceticus, Rhodococcus erythropolis, Rhodococcus* sp., *Paenibacillus* sp., showing an inhibitory effect on all the *Bacillus* species and on *Cellulomonas* sp. The different effectiveness was due to the different sensitivity of the single microbial strain tested. It was effective at very low concentrations (0.05% w/v) and showed an inhibitory effect on Gram-positive strains but not on Gram-negative bacteria.

4.3 LIQ

Licorice (*Glycyrrhiza glabra*) root extract is rich in different classes of phytocompounds, such as phenols (liquiritina, isoliquiritin, liquiritigenina, isoliquiritigenina, glabridina and glabrol), terpenoids and saponins (β-amyrin, glycyrrhizin, glycyrretol, galabrolide, licorice acid), volatiles compounds (benzaldehyde, fenchone, linalool, anethole, estragole, eugenol and hexanoic acid), vitamins (B1, B2, B3, B6, C, E, biotin, folic acid and pantothenic acid), coumarins (glycyrine, umbelliferone, ligcoumarin and herniarin) and mineral content (Öztürk et al. 2018). These are bioactive compounds which anti-bacterial, anti-fungal, anti-viral, anti-tumour, anti-inflammatory, anti-oxidant, anti-allergic, expectorant, anti-malarial and anti-convulsive activities have been widely demonstrated (Abbas et al. 2015). In addition to the innumerable properties of the root extract, phytochemical studies have recently been carried out on the extract of licorice leaves which have shown the presence of classes of phenols and acid compounds such as glabranin, licoflavanon and pinocembrin (Scherf et al. 2012). These compounds are present

only in traces or completely absent in the roots, but which are also known for their antimicrobial and anti-fungal action in plants and men.

The company Trifolio-M GmbH (Lahnau, Germany) has created a formulation from the extract of licorice leaves (LIQ), commonly considered an agricultural waste. The alcoholic LIQ extract, prepared from dried and finely ground licorice leaves, is still in experimental phase and not yet commercialized. Trifolio-M is studying LIQ for its antimicrobial activity against plants pathogenic fungi for application in agriculture. In parallel, LIQ extract is under study by ENEA to verify the possibility of exploiting the antimicrobial properties of this extract towards the biodeteriogens affecting artworks and monuments. The antimicrobial effect of the extract was demonstrated in-vitro towards about 20 bacterial strains (belonging to the phyla *Firmicutes, Actinobacteria* and *Gammaproteobacteria*) and 10 fungal strains (belonging to the genera *Aspergillus, Fusarium, Epicoccum, Penicillium* and *Cladosporium*), isolated from hypogeal environments, frescoes, wall and canvas painting, and included in the ENEA collection of microbial strains. Fungal strains were less sensitive to the extract than bacterial strains; however, the intermediate sensitivity level shown by *Cladosporium* spp. and *Epicoccum nigrum* is a significant result, given that these fungal genera, widespread in hypogeal environments, are characterized by the production of melanins and other deleterious pigments.

4.4 SME 1.11

Arthrobacter xylosoxidans SME1.11 (SUB4060614) is a bacterial strain isolated from the soil of Ingurtosu mining site (Sardinia, Italy). Its cells are non-motile, non-spore forming, Gram-positive, aerobic and rod-shaped. The optimal laboratory growth conditions include 4% of salinity and pH 6 and 28 °C; SME1.11 shows no reducing power, no proteolytic and lipase activities but high pectinase activity, produces siderophores and calcite crystals, and lactate tolerance. This bacterial species belongs to the risk Group 1 that contains non-pathogenic organisms, i.e. do not cause disease in healthy adult humans.

The capacity to degrade pectin and produce siderophores are two characteristics that made this strain a good candidate for biodeterioration control. Further biochemical characterization of SME1.11 properties and its antimicrobial activity are under investigation.

4.5 NopalCap

A natural plant product obtained from the mucilage of *Opuntia ficus-indica* combined with the alcoholic chili extract - NopalCap- is under investigation for its potential application as additive in mortars to reduce biofilm colonization. This product has been studied and carried out in the frame of a bilateral cooperation

project between Mexico and Italy, supported by the Italian Ministry of Foreign Affairs and International Cooperation (Progetto Grande Rilevanza PGR00971). According to the Mexican tradition, the addition of small amounts of *Opuntia* mucilage to lime mortar allowed to a good preservation of ancient mural paintings and other artworks. The bio-receptivity of mortars containing different percentages of mucilage was tested (Persia et al. 2016) and the positive results encouraged us to expand the study to include a further plant substance, already known for its antimicrobial property, i.e. the *Capsicum* extract. The new bio-product called NopalCap was at first tested in laboratory on bio-mortar specimens to verify the capacity to prevent a microbial attack. Then was used as additive in the production of lime mortar and hydraulic mortar for restoration intervention under the supervision of restorers (see Table 11.2).

4.6 Real Cases Application

The LIQ extract was tested at 3% v/v concentration on a painting on canvas for the elimination of mixed patinas including the bacterial species *Bacillus licheniformis* and *Bacillus subtilis*, and the fungal species *Arthrinium* sp., *Aspergillus* sp., *Cladosporium* sp. The treatment was proven effective both in reducing the microbial load and in preventing over time (up to 1 year later) the re-proliferation of microorganisms.

Further evidence of the potential of this formulation in relation to its efficacy towards biodeteriogens was carried out on the frescoes of the chapel XIII of the Sacro Monte di Orta (Novara), affected by a very diffused pink patina, attributable to colonization by strains of the genus *Arthrobacter* (isolated and identified by the researchers at SUPSI, The University of Applied Sciences and Arts of Southern Switzerland, Lugano). The biodeteriogen strains responsible of the pink patina were tested in laboratory for sensitivity to different natural products (NopalCap, BioZ, Liq), in comparison with benzalkonium chloride (Benz) as positive control. After a well diffusion assay LIQ extract was selected and the Minimal Biocide Concentration was quantified as 0.4% v/v, one order of magnitude higher than Benz. After a few tests on the frescoes, to verify the influence of the product on the colour, the application was carried out in the Chapel XIII, during February 2019, on a test area of about 20 square metres, intended for experimentation. The product has been applied by nebulization at the concentration of 1.5% v/v and left on the wall up to 48 h. The treatment did not cause unwanted aesthetic alterations and, after the biocide treatment, the restorers completed the cleaning of the frescoes. The first microbiological monitoring after treatment did not detected the presence of the bacterial species correlated to the pink patina. Monitoring over time is underway to evaluate the preventive or retarding properties of a new colonization, by LIQ extract.

LIQ is also under investigation in the treatment of XVIII century stone sculptures placed in the garden of a private villa near Treviso (Italy) in collaboration with a

Fig. 11.4 Biofilm removal on the stone pedestal of a statue in the Vatican Gardens. The products were applied in cellulose pulp, (**a**) the surface after the cleaning with 1- BioZ; 2- water; 3- LIQ 3%. (**b**) the same surface 18 months after the treatments: the portion cleaned with water (2) shows a new biofilm growth, while the portions treated with BioZ and LIQ are still clean

local restoration firm (Monica Casagrande). After the application, the long-lasting effect on the delay in biofilm growth is being monitored.

BioZ and LIQ products were used in preliminary test on the basement of a statue in the Vatican Gardens. The application was performed absorbing the liquid substances with cellulose pulp before their spreading on the surface previously washed with deionized water. The packs were left in situ for 24 h and then removed (Fig. 11.4a). Comparing the effects on the microbial recolonization 18 months after the treatments (Fig. 11.4b) we observed that the portion used as control, treated with only water, was partially covered with a biofilm, while the two portions treated with BioZ and Liq were still free of colonization.

In collaboration with University of Tor Vergata (Rome) tests on biofilms sampled at the Domus Aurea (Rome) have been carried out (Rugnini et al. 2019) using alcohol extracts from *Glycyrrhiza glabra* (LIQ) and *Capsicum* (CAP), singularly or mixed. Biofilms samples were collected in one of the hypogeal rooms from an undecorated wall and were then homogenized and inoculated in BG11 agar medium. Observations showed that the biofilm was dominated by the cyanobacterium *Scytonema julianum*, often described from other hypogeal environments and known to deteriorate the substratum integrity by dissolution of minerals and the precipitation of calcium carbonate on its sheaths. Within the sampled biofilms some species belonging to *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* and three fungal strains were also identified. The biofilms were treated twice with the extracts at 5 days-interval, and the photosynthetic response of the biofilm was followed for 5 days with a mini-PAM portable fluorometer. Photosynthesis is highly susceptible to this kind of treatment, so measurements of rates were used as a proxy for cell

Fig. 11.5 Biofilm removal on marble artifact at Diocleziano Termae, Rome. (**a**) shows the scheme of the trial, with the product applied in cellulose pulp (P) and in Vanzan gel (V). From left to right: BioZ in cellulose pulp and Vanzan, LIQ in cellulose pulp and Vanzan, NopalCap in cellulose pulp and Vanzan, SME1.11 in Vanzan, C- (water in cellulose pulp) and C+ (benzalkonium chloride 1% in cellulose pulp). (**b**) the result soon after the pack removal

health. Changes in photosynthetic activity of the samples treated with the extracts were compared to control biofilms receiving no treatment. Results showed that LIQ 30% v/v had the highest photosynthesis inhibition potential, followed by LIQ extract 10% v/v. *Capsicum* extract was the least efficient. These initial results will be followed by applications on test areas on the wall and roof surfaces within the Domus Aurea site.

NopalCap was used in preparing a hydraulic mortar to fill the voids in the base of a statue, placed in the patio of the Conservatory of Naples (Italy). A year after the restoration, the mortar is free from biofilm attack, while the neighbouring areas show recolonization of the surface. This experiment had been preceded by laboratory tests on hydraulic mortar specimens also subjected to artificial ageing which gave excellent results and will be included in the Dissertation of a thesis on the restoration of the whole statue (Lorenza Cardone, Accademia delle Belle Arti di Napoli).

In collaboration with the University of Tuscia (Viterbo) and Superintendency for Roman Museum, a broad comparison of the different bio-products under investigation in our laboratory was carried out at Diocleziano's termae (Rome, Italy), on a marble artefact placed outdoor, showing a diffuse biofilm (Fig. 11.5). The surface was examined using the digital microscope Dino-Lite and the biofilm was sampled to study the microbial patina. High-throughput molecular analyses were performed on the biofilm samples: by a preliminary valuation of the data, the biofilm collected before the treatment was dominated by *Cyanobacteria*, mostly belonging to *Nostocales* (76% of the total bacterial diversity) and by fungi belonging to *Ascomycota* (90% of the total fungal diversity).

Particular attention was paid to the application process: the liquid products were immobilized in both cellulose pulp and in Vanzan®NF to test the best procedure for

application. BioZ, LIQ 3%, NopalCap 10% and SME1.11 (only in Vanzan®NF) plus a negative control (water in cellulose pulp) and a positive control (benzalkonium chloride 1% in cellulose pulp) were kept in place for 2 weeks, covered with a plastic and aluminium foil to delay the dry out. As for the application procedure, it was evident that cellulose pulp is not suitable for this kind of products: during the laying of the mixture on the vertical surface, the cellulose pulp did not retain the liquid phase, while the Vanzan®NF gel was able to keep the product into its matrix. After the packs removal, the surface was washed with deionized water, let dry and the results were recorded (Fig. 11.5b): the best biofilm removal and surface cleaning was obtained with SME 1.11 in Vanzan®NF, followed by the pack with water in cellulose pulp. The other bio-products gave less effective results but better when applied in Vanzan®NF; the positive control showed a transient chromatic alteration. Bioluminometer measurement (unpublished data) allowed testing the efficacy of the bio-product on the biofilm control after 7 months. The value on the untreated area was 145,000 RLU, and the positive control (benzalkonium chloride) 8000 RLU while the treated areas show intermediate values, lower than 25,000 for the all the Vanzan®NF -packs.

The visual inspection carried out 11 months after the trial (Fig. 11.5c) showed the persistence of the clean areas for all the treatments except for NopalCap, where the biofilm seems to grow again. Monitoring of the surface is ongoing to evaluate the persistence of the biodeteriogen control.

5 Concluding Remarks

The demonstration cases of bio-cleaning shown and discussed in this chapter provide, together with the research studies carried out by the scientific community, a convincing proof of feasibility of using biotechnology applied to Cultural Heritage. The next step requires the transition from research to production, to make the procedures and proposed products available to the market for restorers. To this end, an effort is needed to define the repeatability of the procedures, the stability of the strains and products when transformed into a marketable product (ex: freeze-dried), to reduce the contact times and to move towards a simplification of the procedures, by selecting multifunctional and versatile microbial strains.

Our experience leads to say with certainty that investigations in the search for new products and procedures for a more sustainable strategy in cultural heritage conservation, especially with a view to reducing or replacing toxic/dangerous products, are flourishing and are widening the field of goods and the issues involved.

Around these issues numerous interests, both scientific and “policy” are gathering. That also implies interventions on the legislative, regulatory and organizational level.

In order for this process to be carried out and to take place in a virtuous way, a joint contribution from the world of Conservation, research, entrepreneurship,

stakeholders, the market and public decision-makers is needed, at European and International level.

The market for new restoration products that are harmless to operators, the environment and works of art must be viewed from the illuminated perspective of sustainability, as it has a very high social, historical and cultural value, although it can be a niche market.

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References

Abbas A, Zubair M, Rasool N, Rizwan K (2015) Antimicrobial potential of *Glycyrrhiza glabra*. J Drug Design Med Chem 1:17–20

Alisi C, Musella R, Tasso F, Ubaldi C, Manzo S, Cremisini C, Sprocati AR (2009) Bioremediation of diesel oil in a co-contaminated soil by bioaugmentation with a microbial formula tailored with native strains selected for heavy metals resistance. Sci Total Environ 407:3024–3032

Barbabietola N (2013) Innovative microbe-based technology for Conservation and Restoration. PhD thesis dissertation, Science for conservation of cultural heritage, University of Florence

Barbabietola N, Tasso F, Alisi C, Marconi P, Perito B, Pasquariello G, Sprocati AR (2016) A safe microbe-based procedure for a gentle removal of aged animal glues from ancient paper. Int Biodeterior Biodegradation 109:53–60

Bastian F, Alabouvette C, Jurado V, Saiz-Jimenez C (2009) Impact of biocide treatments on the bacterial communities of the Lascaux cave. Naturwissenschaften 96:863–868

Beltrani T, Chiavarini S, Cicero DO, Grimaldi M, Ruggeri C, Tamburini E, Cremisini C (2015) Chemical characterization and surface properties of a new bioemulsifier produced by *Pedobacter* sp. strain MCC-Z. Int J Biol Macromol 72:1090–1096

Bio-Based Industry Consortium (2017) Strategic Innovation and Research Agenda (SIRA). https://www.bbi-europe.eu/sites/default/files/sira-2017.pdf

Biocidal Products Directive 98/8/EC (1998). https://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:1998:123:0001:0063:EN:PDF

Biocidal Products Regulation (EU) 528/2012. https://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:167:0001:0123:EN:PDF

Bosch Roig P, Regidor Ros JL, Montes Estelles R (2013) Bio-cleaning of nitrate alteration on wall paintings by *Pseudomonas stutzeri*. Int Biodeterior Biodegradation 84:266–274

Bosch-Roig P, Lustrato G, Zanardini E, Ranalli G (2014) Bio-cleaning of cultural heritage stone surfaces and frescoes: which delivery system can be the most appropriate? Ann Microbiol 65:1227–1241

Colombini MP, Bonaduce I, Gautier G (2003) Molecular pattern recognition of fresh and aged shellac. Chromatographia 58:357–364

Crisci L, Alisi C, Pratelli M, Santamaria U, Sprocati AR (2020) Il Biorestauro: messa a punto di una procedura su misura per l'opera. In: Tra cielo e terra. La Madonna della Cintola di Vincenzo Pagani. Edizioni Musei Vaticani (in press)

Cutraro S (2014) Ricerca di una via biologica per la rimozione di colle viniliche da affreschi: il caso studio degli affreschi delle cappelle di Saint Jean e Saint Martial (Palazzo dei Papi, Avignone). Master's thesis dissertation, Sapienza University, Rome

De Vero L, Boniotti MB, Budroni M, Buzzini P, Cassanelli S, Comunian R, Gullo M, Logrieco AF, Mannazzu I, Musumeci R, Perugini I, Perrone G, Pulvirenti A, Romano P, Turchetti B, Varese GC (2019) Preservation, characterization and exploitation of microbial biodiversity: the perspective of the Italian network of culture collections. Microorganisms 7:685

Desai JD, Banat IM (1997) Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 61:47–64

Devreux G, Santamaria U, Morresi F, Rodolfo A, Barbabietola N, Fratini F, Reale R (2015) Fitoconservazione. Trattamenti alternativi sulle opere in materiale lapideo nei giardini vaticani. In: Abstract, 13th Congresso Nazionale IGIIC -Lo Stato dell'Arte; Turin, Italy. 22–24 October 2015, pp 199–206

ECHA Site (2020). https://echa.europa.eu/substance-information/-/substanceinfo/100.063.544

Fidanza MR, Caneva G (2019) Natural biocides for the conservation of stone cultural heritage: a review. J Cult Herit 38:271–286

Galizzi V (2015) Studio e riassemblaggio della scultura lapidea di Bacco del Museo di Antichità di Torino: ricerca di un equilibrio tra fruizione e conservazione. Master's degree dissertation, Conservation and restoration of cultural heritage, Torino University

Gioventù E, Lorenzi PF, Villa F, Sorlini C, Rizzi M, Cagnini A, Griffo A, Cappitelli F (2011) Comparing the bioremoval of black crusts on colored artistic lithotypes of the cathedral of Florence with chemical and laser treatment. Int Biodeterior Biodegradation 65:832–839

Grimaldi M (2009) Search for new surface-active compounds of microbial origin in view of the development of biorestoration techniques. PhD thesis dissertation, University of Florence

Guiamet PS, Gómez de Saravia SG, Arenas P, Pérez ML, de la Paz J, Borrego SF (2006) Natural products isolated from plants used in biodeterioration control. Pharmacologyonline 3:537–544

Hueck HJ (1965) The biodeterioration of materials as a part of hylobiology. Mater Org 1:5–34

Ledda M (2019) Prodotti biotecnologici per il restauro sostenibile: un caso studio alle terme di Diocleziano, Roma. Bachelor thesis, University of Cagliari, Italy

Martin-Sanchez PM, Nováková A, Bastian F, Alabouvette C, Saiz-Jimenez C (2012) Use of biocides for the control of fungal outbreaks in subterranean environments: the case of the Lascaux cave in France. Environ Sci Technol 46:3762–3770

Mazzoni M, Alisi C, Tasso F, Cecchini A, Marconi P, Sprocati AR (2014) Laponite micro-packs for the selective cleaning of multiple coherent deposits on wall paintings: the case study of Casina Farnese on the Palatine Hill (Rome-Italy). Int Biodeterior Biodegrad 94:1–11

Mora P, Mora L, Philippot P (1984) Conservation of wall paintings. Butterworths, Sevenoaks

Öztürk M, Altay V, Hakeem KR, Akçiçek E (2018) Pharmacological activities and phytochemical constituents. In: Öztürk M, Altay V, Hakeem KR, Akçiçek E (eds) Liquorice: from botany to phytochemistry. Springer, Cham, pp 45–60

Persia F, Alisi C, Bacchetta L, Bojorquez E, Colantonio C, Falconieri M, Insaurralde M, Meza-Orozco A, Sprocati AR, Tatì A (2016) Nopal as organic additive for bio-compatible and eco-sustainable lime mortars. In: Campanella L, Piccioli C (eds) Diagnosis for conservation and valorization of cultural heritage. AIES, Naples, pp 245–251

Pieragostini E (2015) Comparative evaluation of chemical and biological methods for the removal of iron oxide stains from calcareous surfaces. Master's thesis dissertation, Sapienza, University of Rome, Italy

Puškárová A, Bučková M, Kraková L, Pangallo D, Kozics K (2017) The antibacterial and antifungal activity of six essential oils and their cyto/genotoxicity to human HEL 12469 cells. Sci Rep 7:8211

Ranalli G, Alfano G, Belli C, Lustrato G, Colombini MP, Bonaduce I, Zanardini E, Abbruscato P, Cappitelli F, Sorlini C (2005) Biotechnology applied to cultural heritage: biorestoration of frescoes using viable bacterial cells and enzymes. J Appl Microbiol 98:73–83

Rivardo F, Turner RJ, Allegrone G, Ceri H, Martinotti MG (2009) Anti-adhesion activity of two biosurfactants produced by *Bacillus* spp. prevents biofilm formation of human bacterial pathogens. Appl Microbiol Biotechnol 83:541–553

Rodrigues L, Banat IM, Teixeira J, Oliveira R (2006) Biosurfactants: potential applications in medicine. J Antimicrob Chemother 57:609–618

Rotolo V, Barresi G, Di Carlo E, Giordano A, Lombardo G, Crimi E, Costa E, Bruno M, Palla F (2016) Plant extracts as green potential strategies to control the biodeterioration of cultural heritage. Int J Conserv Sci 7:839–846

Rozeik C (2009) The treatment of an unbaked mud statue from ancient Egypt. J Am Inst Conserv 48:69–81

Rugnini L, Ellwood NTW, Sprocati AR, Migliore G, Tasso F, Alisi C, Bruno L (2019) Plant products as a green solution in the fight against biodeterioration of stone monuments. In: Poster 114° congresso S.B.I. (IPSC) - Padova, 4–7 September 2019, p 34

Scherf A, Treutwein J, Kleeberg H, Schmitt A (2012) Efficacy of leaf extract fractions of Glycyrrhiza glabra L. against downy mildew of cucumber (Pseudoperonospora cubensis). Eur J Plant Pathol 134:755–762

Sharma RK, Yenkateswaran NS, Singh SK (1980) Solvent used for the removal of shellac and other accretionary deposits. In: Symposium on mural paintings, archaeological survey of India-ICCROM, Dehra Dun, pp 10–15

Singh M, Balasaheb A (2013) Chemistry of preservation of the Ajanta murals. Int J Conserv Sci 4 (2):161–176

Sprocati AR, Alisi C, Tasso F, Marconi P, Sciullo A, Pinto V, Chiavarini S, Ubaldi C, Cremisini C (2012) Effectiveness of a microbial formula, as a bioaugmentation agent, tailored for bioremediation of diesel oil and heavy metal co-contaminated soil. Process Biochem 47:1649–1655

Sprocati AR, Alisi C, Tasso F (2014) Biotechnology process for the removal of coherent deposits of organic and inorganic origin from materials and works of artistic historical interest. European patent WO2015040647

Sprocati AR, Alisi C, Tasso F, Marconi P, Migliore G (2017) Formule microbiche per l'arte. Kermes 100: 23–26. Nardini Editore

Sterflinger K, Piñar G (2013) Microbial deterioration of cultural heritage and works of art — tilting at windmills? Appl Microbiol Biotechnol 97:9637–9646

Venice (1964) International charter for the Conservation and restoration of monument and sites (the Venice Chart 1964). In: ICOMOS, IInd International Congress of Architects and Technicians of Historic Monuments, Venice