Innovative and Integrated Strategies: Case Studies

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F. Palla, G. Barresi (eds.), *Biotechnology and Conservation of Cultural Heritage*,
DOI 10.1007/978-3-319-46168-7_5

Abstract

In this chapter, case studies related to *biodeterioration*, *bioaerosol*, *biocide* and *biocleaning* are reported. The aim is highlighting the role of biology and biotechnology tools for the preventive conservation of organic and inorganic artifacts, understanding how traditional as well as innovative methods can help the conservationists to develop integrated strategies considering works of art/environment/humans as a dynamic system. Particularly, based on the experience acquired during the researches of *Laboratory of Biology and Biotechnology for Cultural Heritage* (*LaBBCH*), the authors suggest several approaches to reveal and identify biological systems able to induce biodeterioration of cultural assets, also focusing on bioaerosols in indoor environment to assess the risk for historical-artistic collections. Finally, novel bioactive molecules have been applied to perform biocleaning protocols or to control of microbial colonisation, in accordance to conservative restoration procedures and safety for both the environment and operators.

5.1 Biodeterioration of Inorganic Cultural Assets

5.1.1 Fountains

The fountains and all decorative apparatus usually exposed to a water supply and located in outdoor may suffer of several biological colonisation. This is, for example, the case of the biological community revealed in the *Fountain of the Two Dragons* in Palermo (Fig. 5.1a) where the attention has been paid to all discolorations, encrustations and pigmented patinas traceable to past or actual biological systems living onto marble surfaces (Di Carlo et al. 2015). Plants, mosses, insects, rusty patinas, black crusts, dirty deposits, fissures and detachment of material have been investigated. In order to define an adequate conservative restoration procedure, the identification of biodeteriogens has been required and the characterisation performed through an integrated approach based on microscopy

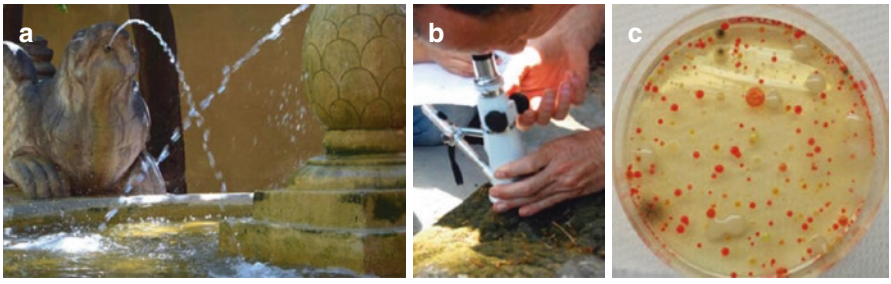

Fig. 5.1 Fountain of Two Dragons: (**a**) water supply in the basin; (**b**) field portable microscope; (**c**) microbial colonies developed on N-agar medium

techniques, in vitro culture and molecular biology investigation. Particularly, pigmented areas have been sampled in order to reveal microbial consortia able to release biogenic pigments. All samplings have been performed using both non-invasive (sterile swab, nylon membrane fragment) and microinvasive methods (micro-scalpel) and supported by portable field microscope (Fig. 5.1b). Macrosystems, collected in different areas of the fountain, have been analysed by digital microscope (Dino-Lite) and stereomicroscope (Wild Heerbrugg); collected bryophytes and insects (or their fragments) have been identified by optical microscope observations (Leica, 40×).

Concerning the microbial community inhabiting the fountain, reddish and pinkish bacteria mostly belonged to *Arthrobacter* and *Paracoccus* genera have been identified (Fig. 5.1c). Molecular biology investigation has been performed through *ad hoc* protocols, including genomic DNA extraction by Genomic DNA Purification Kit (Fermentas). The sequencing has been performed by Eurofins MWG Operon and sequencing service (Germany) and sequence analysis by BLAST platform using the nucleotide databases NCBI-NIH (USA) and EMBL (Germany) (Altschul et al. 1997). Fungal colonies belonging to *Alternaria* spp., *Fusarium* spp., *Cladosporium* spp. and *Phoma* spp. have been revealed. In particular, *Cladosporium* and *Phoma* are considered two of the most important fungal species involved in biodeterioration of urban stone buildings (Sterflinger and Prillinger 2001; Sterflinger and Piñar 2013). Further studies on algae and cyanobacteria will undertake in order to plan a successful conservative intervention.

During *Fontana Pretoria* (Palermo) restoration, a comprehensive study on colonising microbial communities has been carried out. The marble areas showed an expanding and intensifying reddish chromatic alteration. As reported in the literature (Krumbein 2003), these chromatic alterations could be related to bacterial taxa that we identify in this study by molecular investigation (Palla and Tartamella 2007). Non-invasive sampling has been carried out on marble statues by sterile cotton swabs soaked with NaCl-Tween20 solution. Bacteria as *Bacillus*, *Arthrobacter*, *Micrococcus* and *Cellulomonas* have been identified combining *in vitro* culture, SEM observations and molecular investigations, according to protocols carried out in our laboratory. The adequate biocide concentrations (Algophase and Benzalkonium chloride) have been determined by "inhibition bacterial growth" in *in vitro* assays (Palla 2009).

5.1.2 Wall Paintings

Saints Cave (XII-XIV sec., Licodia Eubea, Alia, Sicily) restoration project represented an opportunity for investigating the micro and macro-biological systems colonising this environment, characterised by high relative humidity, percolating water and a continuous air exchange with the surrounding countryside. Among macro-colonisers, *Parametria diffusa* M. et K. and *Adiantum capillus-veneris* identified in the confined environment, microbial systems able to trigger the degradation on frescoes have been revealed combining microscopy and molecular analysis.

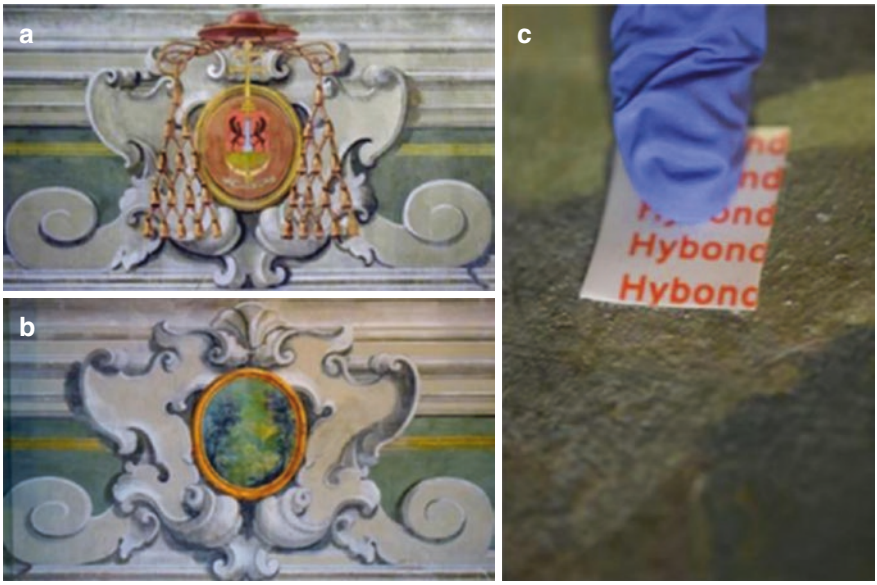

Fig. 5.2 *Borremans* frescoes: (**a**, **b**) investigated areas; (**c**) sampling by nylon membrane fragment (Hybond, Amersham)

Black, green and differently pigmented areas inhabiting the cave wall surfaces have been investigated by CLSM observations (Olympus FV-300 equipped with Argon laser, λ= 488 nm, and Helium/Neon laser, λ=543 nm) at 40× magnification, and the main colonising microorganisms (*Cyanobium*, *Aspergillus*, *Trichoderma*) have been identified by molecular investigation (Palla et al. 2010). The molecular analysis included microbial DNA extraction (Genomic DNA Purification Kit *Fermentas*), PCR (Ready-to-Go *Amersham Biosciences* reaction mixture), sequencing (Eurofins MWG Operon sequencing service) and sequence analysis performed by BLAST platform (Altschul et al. 1997).

Detection of microbial community on fresco surfaces (Fig. 5.2a) has been carried out in *Borremans* hall (Palermo *Diocesan Museum*). The goal of this investigation was to assess the microbial contamination on the painted surfaces at different heights in order to evaluate the microbial "fallout" (microbial sedimentation on a given surface) and how the microbial contamination of aerosol may influence the colonisation of artworks surfaces; for this reason, the monitoring of airborne microbial load was carried out in parallel. Non-invasive sampling (nylon membrane fragments 4 cm^2) has been utilised in order to isolate microorganisms in selected points on fresco surface (Fig. 5.2b). Morphological study of fungal colonies and DNA investigation allowed the identification of microorganisms as *Penicillium chrysogenum*, *Chaetomium globosum*, *Aspergillus versicolor*, *Cladosporium* spp., *Kocuria rosea*, *Nocardiopsis* spp., *Staphylococcus* spp., *Micrococcus luteus* and *Bacillus* spp. The higher microbial load (4 CFU/cm^2) was attributable to *Penicillium chrysogenum*.

Moreover, during the fresco samplings, the surface has been irradiated by UV light, and an elemental analysis has been performed by portable X-ray fluorescence (XRF) instrument (Drand and Laval 2009).

5.2 Biodeterioration of Organic Cultural Assets

5.2.1 Paper

Especially for those objects made of paper, the detection of fungi or bacteria is a fundamental step in order to prevent and control the paper decay. The main foresight is the occurrence of a non-destructive sampling; nitrocellulose and nylon membranes have been found to be useful for this purpose. Moreover, a constant monitoring of specific microbial strains enables the evaluation of the most appropriate conservation strategies.

In this scenario, the identification of bacteria and especially of fungi is fundamental, since they may be considered the main cause of chromatic alterations, originating spots of different colours. It has been observed that the presence of fungi can cause also structural alterations that means of fragility of paper or their partial/total destruction. It is necessary to keep in mind that a non-invasive sampling allows the isolation of bacterial and fungal community in archive, libraries and paper document repositories without damaging the cultural items. Particularly, a study of microbial deteriogens has been carried out on documents, drawings and photos. After sampling, the membranes have been put onto nutritive media (Sabouraud or N-Agar) in order to favour microbial growth (Palla et al. 2015). The results obtained from culture-dependent method, molecular analysis and microscopic identification have confirmed the presence of fungal taxa, including *Cladosporium* spp., *Penicillium* spp. and *Aspergillus* spp. typical fungal infections in libraries, colonising documents made of paper (Sterflinger and Piñar 2013). Differently pigmented areas were sampled by nylon membranes (Fig. 5.3a) to perform culture-dependent analysis (Fig. 5.3b). The morphology of isolated fungal strains has been determined in parallel with molecular investigation, performed through the amplification of 18S-ITS1 rDNA target sequences (Palla et al. 2011). The *Penicillium* has been found as dominant genus, showing a very high microbial load; actinomycetes and bacteria (*Bacillus* and *Micrococcus*) were also found.

5.2.2 Leather and Parchment

Microbial colonisation on several ancient parchment items has been investigated. Non-invasive sampling has been performed using sterile nylon membrane fragments, which were then inoculated into nutritive solid media. The surface sampling has been carried out both on previously treated by fumigation (5 years before) and not treated parchments. The study has sought to highlight the antimicrobial efficiency of fumigation treatment over time. However, the results showed that the

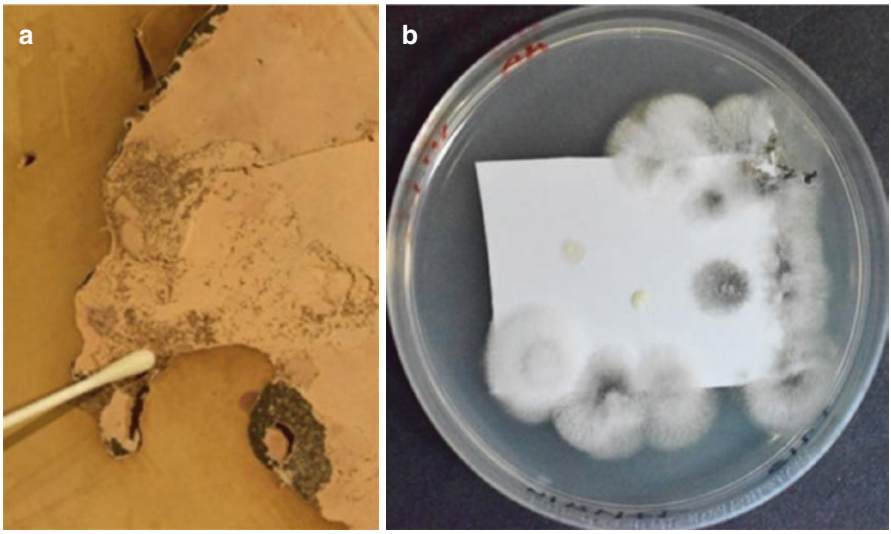

Fig. 5.3 (**a**) Paper affected by microbial decay; (**b**) wide fungal growth (*Penicillium* spp.) on nylon membrane fragment into nutritive medium

disinfection treatment has not prevented microbial recolonisation, allowing the growth of very dangerous fungal strains, such as *Penicillium* and *Alternaria*. Furthermore, the presence of *Micrococcus* spp. and *Staphylococcus* spp. is probably attributable to wrong handling parchments by operators and users.

5.2.3 Wood

The investigation protocols developed in our studies (Palla et al. 2013; Palla et al. 2015a; Perez et al. 2017) have been applied to a wide range of wooden findings and artefacts, in order to shed some light on microbial degradation phenomena, indispensable for correct conservation and restoration strategies.

Archaeological waterlogged wood, part of a bronze rostrum (*Rostro di Acqualadroni*) dated from third century BC (Santamaria et al. 2009), have been analysed by an integrated approach. Particularly, cross and radial sections of wood samples have been observed by light microscopy (magnification range 10.5–40×, reflected light and 40–200× transmitted light) and identified as *Pinus* sp. SEM. The SEM investigation has been utilised to assess the changes in the wood anatomical structure and to deal the state of conservation of submerged wood, evaluating the decay traceable to microbial activity (Blanchette 2000). The SEM micrographs (Fig. 5.4) revealed a specific cell wall alteration attributable to bacterial activity and abundant pyrite (FeS_2) framboids (present as single structure or clustered). The presence of sulphur compounds in archaeological waterlogged wood indicates both long-term burial in anoxic environment and colonisation by sulphate-reducing

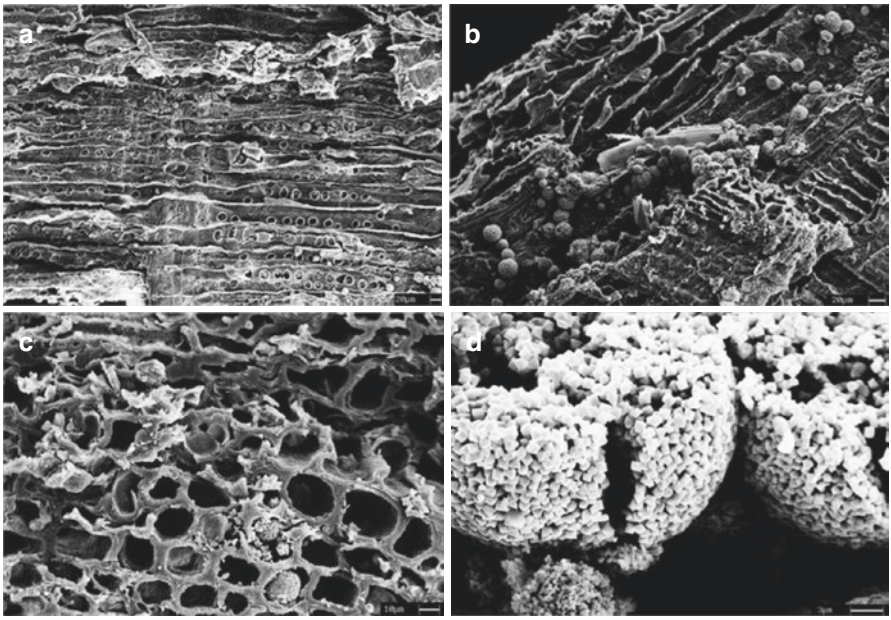

Fig. 5.4 SEM micrographs of wooden thin sections. (**a**, **b**) radial wood structure; (**c**) pyrite framboids in tracheids; (**d**) magnification of a single framboid

bacteria (Palla et al. 2014). Moreover, using culture-independent methods, iron-oxidising bacteria and iron sulphate reducers, respectively, *Marinobacter* sp. and *Desulforudis audaxviator*, have been identified. Bacteria with ligninolytic or cellulolytic activity belonging to *Cellulomonas*, *Bacillus*, *Pseudomonas*, *Xanthomonas* and *Sphingomonas* genera were also identified.

5.3 Bioaerosol in Confined and Semi-confined Environments

Confined and semi-confined environments show peculiar temperature and relative humidity values, fluctuating between day and night, where microbial metabolites are excreted and gaseous and particulate pollution are present, all phenomena that play an important role in the artwork deterioration. Heating, air-conditioning or ventilating system (HVAC), building structures, outside air exchange or huge numbers of visitors generate potential risk factors. In order to establish exposure thresholds, microbial fraction of air samples must be isolated, by means of active or passive sampling.

In the last years, different indoor cultural heritage environments were investigated by the measurement of environmental and microenvironmental parameters that are essential for a correct study of the site and for its correct conservation in accordance with the typologies of material stored or exposed in.

Table 5.1 Airborne microorganisms (bacteria and fungi) identified in crypt and museum environments by active and passive method

Environment	Sampling method	Bacteria	Fungi
Crypt	AirPort MD8	*Nocardiopsis* spp. *Staphylococcus* spp.	*Alternaria* spp. *Cladosporium* spp. *Penicillium* spp.
	Sedimentation	*Arthrobacter* spp. *Bacillus subtilis* *Micrococcus luteus* *Paracoccus* spp. *Staphylococcus* spp.	*Alternaria* spp. *Chaetomium* spp. *Cladosporium* spp. *Penicillium chrysogenum* *Phoma* spp.
Museum	AirPort MD8	*Bacillus* spp. *Paracoccus* spp. *Staphylococcus* spp.	*Aspergillus* spp. *Aspergillus niger* *Cladosporium* spp. *Penicillium* spp. *P. chrysogenum* *Scopulariopsis* spp. *Trichoderma* spp.
	Sedimentation	*Arthrobacter* spp. *Bacillus* spp. *Micrococcus* spp. *Nocardiopsis* spp. *Paracoccus* spp. *Pseudomonas* spp. *Staphylococcus* spp.	*Aspergillus* spp. *Cladosporium* spp. *Penicillium* spp.

During these studies, we emphasise the use of integrated approach to outline the microbial contamination in indoor air of Sicilian cultural sites (Table 5.1) and its role in both artefact deterioration and health effects on visitors/operators. In order to detect and characterise the microbial contamination, the sampling of surface (swab, nylon membranes) and aerosol (active, passive) has been performed to obtain complete information on microbial consortia. Particularly for hypogeal environments, their structural features mainly attributable to the lithic substrates and the different air exchanges with the outside may enhance the growth of several microbial taxa. When the environment is characterised by stable environmental parameters and reduced number of visitors, few amount of microbial taxa may be isolated (Di Carlo et al. 2016).

In museums and archives, bioaerosols generally have a different composition in respect to hypogeal environments. Usually, in archives/libraries and museums/galleries, different artefacts very often composed of organic-based materials, ideal substrates for microbial colonisation, are stored or exposed.

In these environments, the thermo-hygrometric parameters are quite stable, but frequently the absence of an adequate air condition systems or air ventilation may compromise the conservation of cultural assets. Moreover, the number of visitors in museums and galleries strongly influences the environmental parameters. Several environmental fungal and bacterial strains have been isolated using both passive (sedimentation method) and active sampling methods (Fig. 5.5).

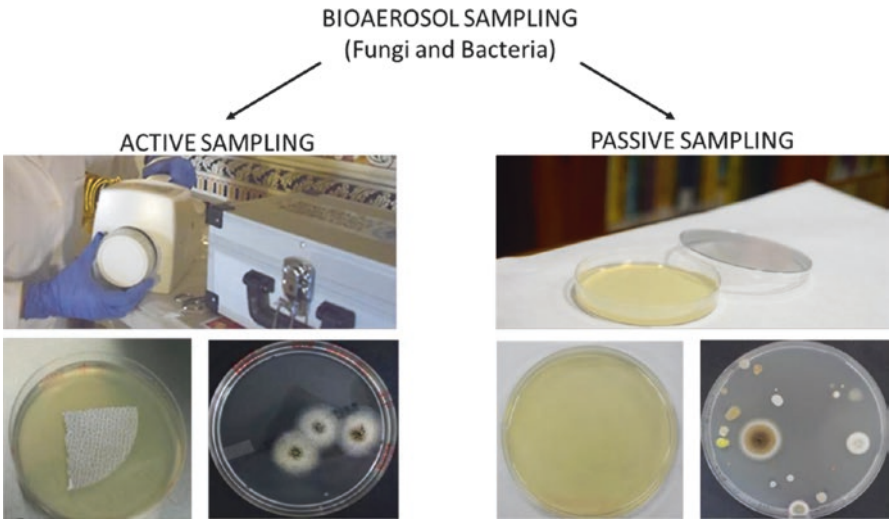

Fig. 5.5 Bioaerosol active sampling (*left*) by AirPort MD8 equipped with gelatin filters. The filter is layered onto nutritive media in which microbial colonies (after incubation time) have grown. Passive sampling (*right*) by gravitational sedimentation onto nutritive media, microbial growth after 16–72 h of incubation at 30 °C

For active sampling, a portable sampler (AirPort MD8, Sartorius) with a flow rate of 100 L/min, equipped with sterile disposable gelatin filter, is used (Palla 2012). Collection filters have been utilised for in vitro culture, laying it onto solid culture media, or dissolved in 1X TE solution (10 mM TRIS–HCL pH7.5/1 mM EDTA) and utilised for the direct extraction of microbial genomic DNA and for molecular biology investigations.

5.3.1 Loop-Mediated Isothermal Amplification of DNA (LAMP)

We propose the use of a new molecular biology system, based on loop-mediated isothermal amplification (LAMP), which allows in situ genetic study without sophisticated laboratory instrument, applicable in cultural heritage field and useful to assess the presence of specific microbial taxa in the indoor environments. This technique, more sensitive and specific than qualitative PCR (Notomi et al. 2000), allows a rapid amplification of nucleic acid at constant temperature, performing tests directly on the site (Yassen et al. 2015). The proposed system (patented by Enbiotech s.r.l) is composed of a portable instrument and a set of ready to use reagents developed for this specific application. The test foresees a rapid preliminary nucleic acid extraction from the sample, genetic amplification using LAMP technology, detection of the fluorescence emitted from the sample and automatic interpretation of the results using the portable instrument (Fig. 5.6). Airborne microbiological particles are collected using AirPort MD8 air sampler, equipped with

Fig. 5.6 PCR-LAMP technology. ICGENE portable mini instrument. The amplification profiles specific for bacteria or fungi cells can be directly followed on wireless connected tablet. ICGENE is a high sensitive, specific and easy-to-use in vitro diagnostic device able to perform the amplification of DNA target sequences in few minutes. The LAMP technology includes a portable fluorescent reader by measuring of light emission of analysed sample, which provides step-by-step assistance in performance of analysis by the use of an intuitive front-end (tablet and app)

sterile gelatin filter, in order to extract the whole microbial genomic DNA, performing the LAMP test by specific primers for a chosen model pathogen microorganism.

In our hypothesis, this molecular device provides an important contribution to the development of innovative protocols to evaluate *in situ* the composition of microbial community, representing an improvement to PCR-based assays and microbiological analysis.

5.4 Biocleaning: Bioremoval

Selected enzymes or viable bacterial cells have been applied for biological removing of undesired matter (organic layers) or crusts (sulphate, nitrate), respectively, from artwork surface. These approaches are based on the enzymatic hydrolysis of layer or on the use of deposits as carbon source or electron for viable bacterial cells (Palla et al. 2016; Alfano et al. 2011).

In the last decades, biomedical and pharmaceutical research pay particular attention to the so-called Blue Biotechnology, in order to isolate and characterise bioactive molecules from marine organisms (from fish, sponges, jellyfish, marine invertebrates, micro-algae) useful in food industry and for biomedical application. Particularly, cold-active molecules are interesting in relation to their stability and activity at low temperature (<30 °C). Different sets of novel enzymes extracted by marine organisms with protease and esterase activity have been tested, in order to

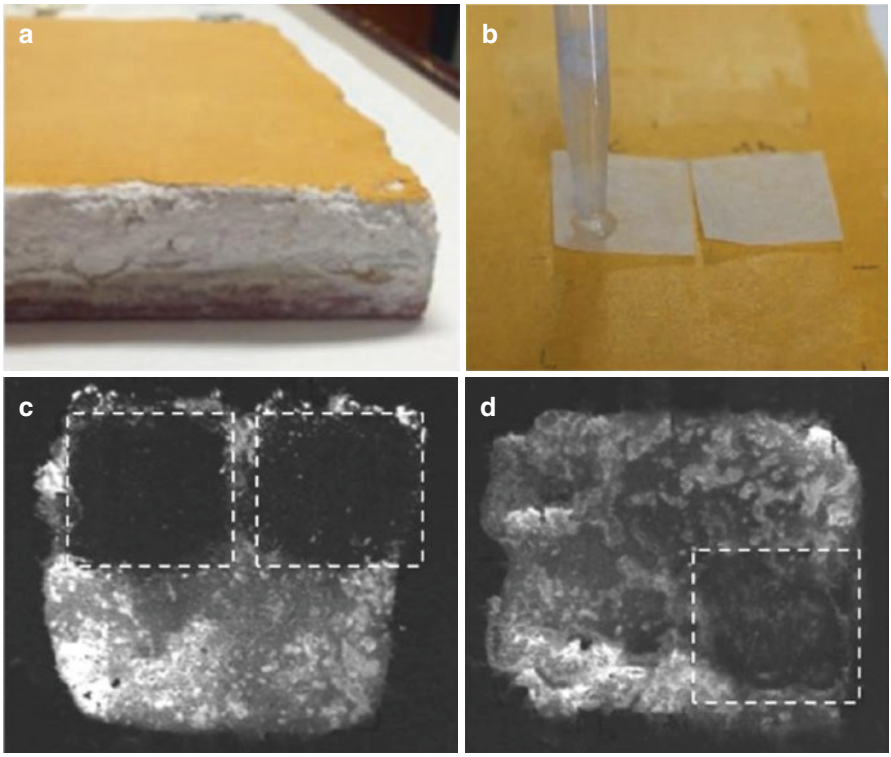

Fig. 5.7 (**a**) Fresco specimen, biocleaning tests by BMP enzyme gelled (Klucel G) solution applied, (**b**) through Japanese paper, on animal glue artificially aged layer; (**c**) efficiency of biocleaning checked by UV fluorescence irradiation by BMP, after 5′ (*left outlined area*) and 10′ (*right outlined area*); (**d**) control test (on the *bottom right*) performed by buffer gelled solution lacking of BMP

remove in a selective way undesirable or altered layers from artwork surfaces, previously characterised by high-performance liquid chromatography. The enzymatic cleaning has been performed to remove layers from artworks or ad hoc stratified laboratory specimens, applying the enzymatic gelled solutions (Klucel G, gellan gum gel, carboxymethylcellulose) in order to perform a selective and controlled action (Barresi et al. 2015; Chillè et al. 2014). Bioactive molecules with protease activity (BMP) have been applied to clean up protein layers from wax and papier-mâché sculptures or to remove an old *velinatura* (Japanese paper bonded with animal glue) from oil painting surfaces (Palla et al. 2015b, 2016). Moreover, animal glue layered on fresco specimen (Fig. 5.7a, b), artificially aged (for 2.200 h, UV-A 300–400 nm; $T = 22 \pm 5$ °C; RH = 60–65 %), has been successfully hydrolysed. Particularly, the efficiency of cleaning has been confirmed by UV fluorescence, 5–10 min of application at 22 °C (Fig. 5.7c), a less deep cleaning result in control sample (Fig. 5.7d). This removal effect is probably attributable to the Klucel G (Wolbers 2007).

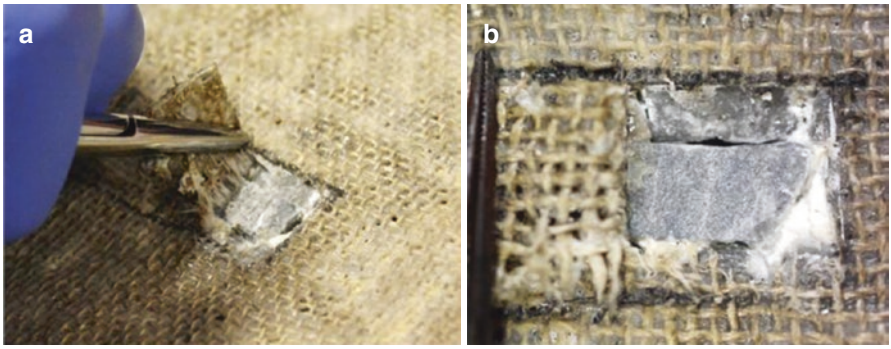

Fig. 5.8 Mosaic laboratory specimen: (**a**) canvas layer has been easily detached after 30 min of treatment; (**b**) Paraloid residues completely removed from tesserae after additional 10 min of BME application

Recently, the research for other bioactive molecules has allowed the isolation of BME (bioactive molecules with esterase acivity).

In order to detach the canvas layer glued by Paraloid B72, a gelled (Klucel G) BME (1mg/ml) solution has been applied on canvas layer; after 30 min, the layer (naturally aged for five years) has been successfully removed (Fig. 5.8).

These enzymes show some important advantages, such as the possibility to work safely, at room temperatures (19–26 °C) and in a quick way (5–30 min), without heating the enzyme solutions or the surfaces on which they have been applied.

Regarding the bioremoval of undesired surface alterations, like black crusts, *Desulfovibrio vulgaris* viable cells have been particularly utilised for the removal of sulphate crusts from marble polychrome bas-relief, representing the Eternal Father exhibited at the *Regional Gallery of Palazzo Abatellis*, Palermo. The viable bacteria (*Micro4Art solfati*© produced by *Micro4you* S.r.l.) have been applied on deposit present on surface as a gelled solution. Even if the application of bacterial gel solution has been difficult, mainly due to the vertical location of the artefact and its complex geometry, the effectiveness of bioremoval of black crusts (previously characterised by XRF and FTIR analysis) was evident from the first application. Moreover, the successful removal of sulphate crusts has been confirmed by XRF analysis performed on a cleaned area (Martino et al. 2015). An important feature of this cleaning method is the speed and ease of use, and considerable importance is represented by the guarantee of a gradual and selective cleaning action and safety for the operators (non-pathogen bacteria), environment and work of art.

5.5 Microbial Growth Control

5.5.1 Bioactive Molecules from Marine Organisms

Due to the well-known antimicrobial properties of several marine organisms, the interest in application in pharmaceutical and biomedical fields (Blue Biotechnology) increases so fast.

In this scenario, bioactive molecules with antimicrobial activity (BMAs) extracted from marine invertebrates have been tested to control bacteria and fungi colonisation in laboratory specimens in order to inhibit the microbial growth on cultural assets. These BMAs have been applied in antimicrobial in vitro assays against bacteria and fungi isolated from colonised organic materials and from organic products usually used in restoration procedures. In this work, three sets of BMA molecules have been assayed on canvas and glue specimens, evaluating the control of microbial contamination (Mulè et al. 2014). In particular, the minimum inhibitory concentration (MIC) and biocide or biostatic activity has been determined by microdilution method using 96-Well Microtiter (Barresi et al. 2015). All tests have been carried out using methylparaben and benzalkonium chloride as controls.

In our hypothesis BMA molecules represent a valid alternative to traditional biocide products, safe for works of art, restores and environment, in accordance with sustainable restoration projects.

5.5.2 Plant Extracts

In the recent years, with the aim to develop alternative products to traditional biocides, several studies have been focused to various natural products with potential antimicrobial activity. Particularly, the use of plant extracts has been recognised in cultural heritage field in order to control and prevent the colonisation by microorganisms or by insect pests (Borrego et al. 2012).

Antimicrobial activity of three different plant products, *Tea tree essential oil*, *Nepeta nepetella* L. and *Allium sativum* L. extracts, has been assayed by *agar disc diffusion*, *well plate diffusion* and *microdilution* methods against bacteria and fungi, previously isolated from colonised artefacts and identified by microscopy and molecular biology investigations (Rotolo et al. 2016). Establishing for each bacteria (*Bacillus subtilis*, *Micrococcus luteus*) and fungi (*Penicillium chrysogenum*, *Aspergillus* spp.) the MIC and the inhibition halo (diameter in mm), the different susceptibility to the plant extracts has been observed during antimicrobial assays. Results showed that the most quick method has been *agar disc diffusion* (Fig. 5.9), allowing us to hypothesise the use of these plant products as natural biocides in the control of biodeterioration of cultural assets, respecting human health and the environment.

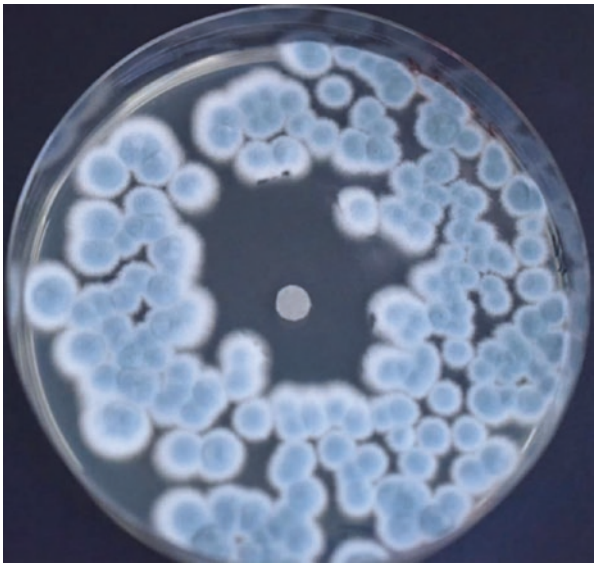

Fig. 5.9 Disk diffusion method using *Allium sativum* extract as antimicrobial agent against *Penicillium chrysogenum*, confirmed by the growth inhibition halo (Rotolo et al. 2016)

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