Microbe-Based Technology for a Novel Approach to Conservation and Restoration

Changes both in the human perception of the environment and in conservation thinking, that have evolved following the Venice Charter (1964), require changes in the approach to scientific and technological research for cultural heritage. Bio-based methods are meeting those needs, presenting some advantages over the traditional chemical-physical methods: low environmental impact, absence of toxic effects for operators, selectivity for the weathered material, safety for the artwork and economical costs. Ongoing activities at ENEA in microbe-based technology address problems still lacking solution or needing improvement with respect to costs, feasibility and safety, both for operators and artworks; among them, the removal of shellac and adhesives from different materials composing artworks or historical documents. An overview of diagnostic and bio-restoration activities is presented

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Introduction

In recognition of the fragile nature of our cultural heritage and its invaluable legacy, which is integral to our future, European policy has sought to identify the best, most sustainable means of conserving our artworks. The Venice Charter (1964) identified a number of key conservation principles relating to minimum intervention, reversibility, repeatability and retreatability, providing, in this way, a framework for deciding on acceptable and unacceptable conservation interventions. These conservation principles are not static, but have evolved over time, as a consequence of both the internal development of conservation as a profession and the changes in the human perception of the world, and of the environment in particular. From the middle of the last century, a widespread opinion in unlimited progress and prosperity led to believe in the principle of human universality. Fifty years on, our perspective has changed to one in which we recognize the complexity and dynamism of the world and the variety of species and cultures it contains. So perspective has changed to a global rather than a universal one, diversity is now a key issue, whether cultural or ecological, and sustainability has become a social, economic and political force. This perspective

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accepts not only the complexity of life, but also that it is impossible to control everything (European Parliament; Directorate-general for research - 2001).
Through all these changes, the principles of conservation activity have been reinterpreted. So, whilst in the past we would have expected to achieve the total control of the environment, we are now prepared to accept the principle of minimum intervention, the notion of the life-cycle of materials, including heritage materials, and the development of the concept of ''acceptable levels of damage''. The principles of reversibility and/or repeatability have been openly discussed and have been replaced by the principles of compatibility and retreatability, which represent a more sustainable conservation strategy. Compatibility requires that treatment materials do not have negative consequences, and retreatability requires that the present conservation treatment will not preclude or impede future treatments. These principles are considered more sustainable because they are more realistic and enable future treatments to take advantage of progress in scientific knowledge, whereas preventive conservation is closely associated with environmental sustainability.
These changes in conservation thinking require changes in the approach to scientific and technological research for cultural heritage (European Parliament; Directorate-general for research - 2001). In the last decade, chemistry, physics and material science played an important role in many aspects of cultural heritage conservation, but today the scene is dominated by biotechnology and applied microbiology, which contribute to the development of new methods, both for a correct biodeterioration diagnosis of weathered historic artworks (Fernandes 2006) and for the development of bio-based restoration techniques for cleaning and consolidation applications (Palla et al. 2006). The development of bio-based techniques took a great advantage from the scientific knowledge gained through research projects funded by the European Community in the Fifth Framework program (2011 EUR 24490 Directorate-General for Research and Innovation Environment). The application of biotechnology for improving the conditions of deteriorated artworks has been recently developing (Webster & May 2006). The biocleaning of frescoes (Ranalli et al. 2005), the removal of black crusts and salts from stone (Heselmeyer et al. 1991, Cappitelli et al. 2007), the bio-consolidation of carbonatic materials (Tiano et al. 1999, Rodriguez-Navarro et al. 2003, Sprocati et al. 2007) represent examples of where microbial technology may contribute to the innovation of conservation.

Ongoing Activities at ENEA: Microbial Technology for Open Issues

The application of physical and chemical techniques to the field of Cultural Heritage has a long history inside ENEA, whereas the application of microbial biotechnology is recent. Our research addresses problems still lacking solution or needing improvement with respect to costs, feasibility and safety both for operators and artworks. Biotechnologies present advantages over the traditional chemical-physical restoration methods: low environmental impact, absence of toxic effects for operators and human health, selectivity for the weathered material, safety for the artwork and economical costs (Webster & May 2006). The activities started in collaboration with the Superintendency for Southern Etruria, on the occasion of the restoration of the Etruscan hypogeum of Mercareccia, located in Tarquinia necropolis of Monterozzi, declared World Heritage in 2004 by UNESCO. The work involved the characterization of large deteriorated areas of the tomb walls, once decorated with reliefs and paintings and now almost entirely disappeared. Microbial characterization, carried out using both classical cultivation and molecular techniques, allows understanding which microorganisms are naturally able to colonise that monument in the course of time, providing a reasonable base for the development of monitoring protocols for the prevention of the biodeterioration over time. At the same time, it allows isolating microbial strains, that can be studied and used for bio-restoration applications. Activities continued on bio-restoration, identifying some critical problems still open, such as the removal of shellac, which is a serious problem for valuable mural paintings, and the removal of adhesives from paper documents.

Microorganisms play a dual role, as a single function can be harmful or beneficial, depending on the circumstances. They can be responsible for the destruction of cultural heritage assets, but this "destructive" action can be used for the biological removal of deteriorated superficial patinas, or even converted in a 'constructive' action, able to induce the precipitation of calcium carbonate deposits on carbonatic materials. Beneficial use of microorganisms for cultural heritage applies both to bio-cleaning and to bio-consolidation, which represent the main bio-restoration application fields.

Metabolic capabilities of our interest in bio-restoration are the ability to precipitate calcite and, conversely, to dissolve carbonate patinas; the capacity to biodegrade resins and glues; the ability to produce biomolecules with surfactant or emulsifying properties. Preferably, the strains should be non-spore-forming, to be sure not to leave latent forms after treatment, and non-pathogenic. The about 120 strains isolated from the tomb walls are now part of the laboratory strains collection "ENEA-Lilith", containing numerous bacterial strains of different environmental origin, suitable for applications of bio-consolidation and/or bio-cleaning. The activities are briefly illustrated in the following; scientific details are exposed in published papers (Sprocati et al. 2007, Sprocati et al. 2008). Detailed information and a historical excursus on studies carried out on the addressed restoration problems may be examined in depth in the doctoral dissertation of Barbabietola (2012).

Bio-Consolidation

The research into a new approach which uses bio-carbonates, more similar to the nature of the stones treated, derives from the necessity to get over some of the drawbacks of the traditional consolidation treatments: incompatibility between the stone and the material applied, low penetration, scant solubility and high degradation, in particular for organic products.

All this has motivated several research groups to carry out numerous experiments, which have led to the development of two patents (Adolphe et al. 1990, Castanier at al. 1995) and to the European project BIOREINFORCE. The first applications of a bio-mediated consolidation were based on the use of Organic Matrix Macromolecules (OMMs) extracted from the shell of a mollusc (Mytilus californianus) (Tiano 2005). Recently, Jroundi F. et al. (2010) demonstrated that the application of an appropriate nutritional medium to non-sterilised porous limestone (calcarenite) in the sixteenth century San Jeronimo Monastery in Granada, Spain, led to the formation of a coherent cement of bacterial calcium carbonate within the stone pores, highly efficient for the consolidation of carbonate stones like calcarenite, due to the presence of native calcinogenic bacteria.

From the Etruscan tomb of Mercareccia we isolated several bacterial strains capable of precipitating calcium carbonates (bio-mineralizers), allowing for the establishment of a collection of strains producing crystals of different colours and sizes, that may be useful for applications to different colour gradation stones (Fig. 1). Some of them have not so far been described for calcinogenic ability. On the basis of the rate of crystals formation on colonies grown on agar medium, as well as of a visual assessment of the amount of crystals purified from it, a few strains have been chosen for a trial of bio-consolidation on the Pietra di Lecce, by in vivo application. The strain TSND 13 (genus Rhodoccoccus) has performed best, considering the reduction of water absorption by capillary action (about 20% vs. control), the alterations in the colour of the stone, the type of biofilm formation and the type and amount of precipitated crystals. This bacterial genus had not been studied for similar applications yet and, compared to the strains usually used in these studies, it has the advantage of being non spore-forming and so easy to eliminate at the end of treatment.

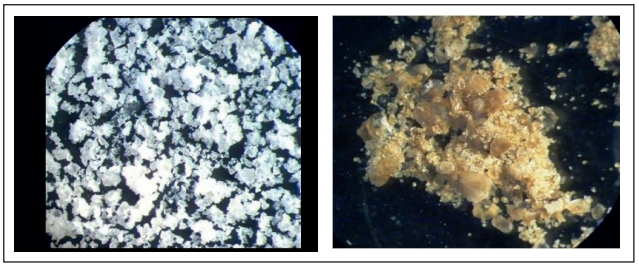

FIGURE 1 Crystals of calcite produced by colonies of two different bacterial strains (stereomicroscope 50x)
Source: Sprocati el al, 2008

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Bio-Cleaning

Removal of Shellac Films from Mural Paintings

Shellac is a natural resin of animal origin, produced from the glandular secretion of an Indian scale insect, Kerria lacca, which lives on numerous trees from the East Indies. On the molecular level, shellac is a complex mixture made of mono- and polyesters of hydroxy aliphatic and sesquiterpenoid acids (Colombini et al. 2003). 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 (Bridgland, 2008, Sharma et al. 1980).

The Ajanta caves had been forgotten and, after re-discovering in 1819, they were further subjected to erosion and human vandalism. In 1920-1922, serious restoration work was initiated by an Italian Conservator L. Cecconi and his associates, applying the white quality of shellac diluted in alcohol or turpentine oil as a preservative coat to protect the paintings (Sharma et al. 1980). Around 1970-1980, the work was continued by the staff of Hyderabad state, who repeatedly and liberally applied shellac, probably with the aim of brightening the paintings (Mora and Philippot, 1984).

Although shellac showed good penetration features, adhesive properties and resistance to biological attacks, the resin had a tendency to yellowing and darkening, becoming hard, brittle and insoluble, because of polyesterification reactions accelerated by high temperatures (Fig. 2). Therefore, cleaning operations were necessary to re-establish the visual perception of the paintings. In the last thirty years different cleaning procedures, which have involved the use of various solvents, have been carried out. Colour loss, white crusts and surfacing of soluble salts appeared after each treatment. Recently, an investigation into Cave 17 highlighted the fragility of the paint layers and the worsening of lacunae over time. Moreover, general phenomena of abrasion of the pictorial film, ascribable to cleaning operations using over-abrasive methods, are evident in specific areas on the different walls (Giovagnoli et al. 2008).

From the cited example, it is evident that, even where a procedure of solubilisation of the resin could be set up, further deterioration processes might occur. The result is an extreme difficulty in the removal of the resin and the impossibility of carrying on successive restoration procedures, compromising the artwork conservation. Moreover, the use of chemical solvents in confined spaces, such as caves, can have harmful effects for conservationists.

Shellac Biodegradation

To our knowledge from the literature, there are no previous studies on the ability of microorganisms to use shellac as a carbon source, degrading it into less complex molecules.

Following a screening, performed on numerous strains of laboratory collection "ENEA-Lilith", using the Biolog systemTM, we selected three bacterial strains, CONC 11 (genus Pseudomonas), CONC 18 (genus Achromobacter), and LAM 21 (genus Acinetobacter), capable to grow over three orders of magnitude higher than the concentration of bacterial inoculum

FIGURE 2 Images of an Ajanta painting (a) and of a detail (b), showing the worst conservation conditions
Source: http://www.saigan.com/heritage/painting/ajanta/ajanta.html

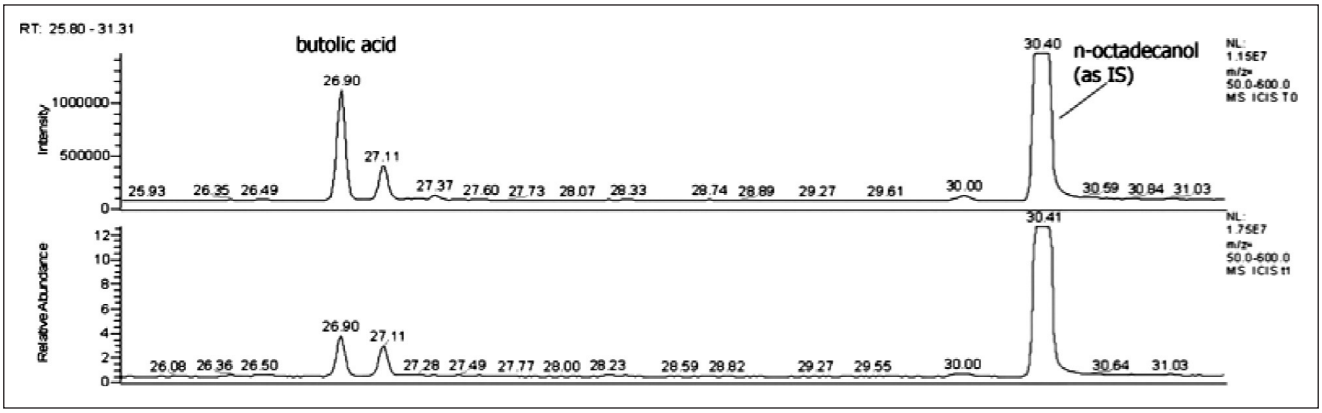

FIGURE 3 Gas Mass analysis of Shellac composition at baseline and after 72 hours (top-down) of growth of the strain CONC 18
Source: Doctoral thesis Barbabietola, 2012

within 24 hours, using shellac as sole carbon source. The rate of use is a key parameter in such a case, as an application over an extended period of time may pose a risk to the integrity of the artwork. In our batch experiments a partial degradation of fresh shellac was observed. Thanks to gas-mass analysis and the novel extraction procedure we developed, it has been shown that the bacterial growth was supported by the butolic acid, the more hydrosoluble fraction of shellac, reduced by 80% after 72 hours (Fig. 3). Given the macromolecular nature of shellac, work is in progress to increase the degradation action, by developing a microbial formula with a higher degradative power, thanks to the metabolic networks individually provided by the different strains. Pending the development of the microbial formula, specimens of mural paintings, partly coated with a thin film of a shellac solution, were purposely prepared for in vivo testing that will be carried out. The specimens are now under artificial weathering, at environmental conditions of 34 °C and 90% R.H, which simulate the annual average climate of the Ajanta caves.

Removal of Animal Glues from Paper Materials

Animal glues are natural polymers derived from the bones, skins, tendons and cartilage of mammalians or fishes. These glues may exhibit varied physical, chemical and mechanical properties, depending on their origin and method of preparation. Animal glues are mainly made up of collagen, which is an insoluble fibrous protein consisting of long molecules composed of naturally occurring amino acids that are linked in a specific sequence by covalent peptide bonds. Collagen is insoluble in cold water and is transformed into soluble gelatine by denaturation in hot water, a process of critical importance for the performance of the resulting glue. 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 will affect 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 artworks. For centuries, cleaning was the most common, indeed the sole form of paper restoration, as the large number of methods, products and 'formulae', handed down to us, testifies. The choice of a cleaning method depends

on the degree and extent of dirt and stains, each requiring specific treatment. Mistaken diagnoses can increase damage through the prescription of the wrong cure (Crespo et al. 1984). These methods, together with more recent recipes, can be divided into four groups: 1) Mechanical methods, 2) Cleaning with non aqueous solvents, 3) Bleaching, 4) Washing enhanced with the use of specific substances, such as detergents, colloidal agents or enzymes.

Each method shows drawbacks. 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. Moreover, the successful enzyme applications described in the literature are mainly directed at the removal of starch paste (Schwarz et al. 2003).

These considerations have led us to develop a method for the removal of animal glues from paper, which combines efficacy of treatment with ease and feasibility. For this purpose, we use bacterial cells that do not require restrictive operating conditions.

Cervione glue and rabbit skin glue were chosen to carry out degradation trials because they were the most used in cultural heritage conservation. Following a screening, five strains were selected from the laboratory strains collection "ENEA-Lilith", able to grow on glue/ glues as sole carbon source. None of the selected strains had cellulolytic activity, which would render them unsuitable for application on paper material.

Original paper samples, kindly supplied by the National Institute for Graphics, representing the back supports of ancient prints from a historical volume of prints from the collection of the Institute, have been treated with bacteria. The strain TSNRS 15 of the genus Ochrobactrum, deriving from an Etruscan hypogeum, was selected as a good candidate, because it was able to grow on both glues within 24 hours. This research applied for the first time this bacterial genus in a process of paper bio-cleaning. The vulnerability of the support (paper) requires much care in developing a gentle procedure; the bio-cleaning treatment was thus performed with bacteria immobilized in an agar gel. The treatment showed its efficacy already after 4 hours 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

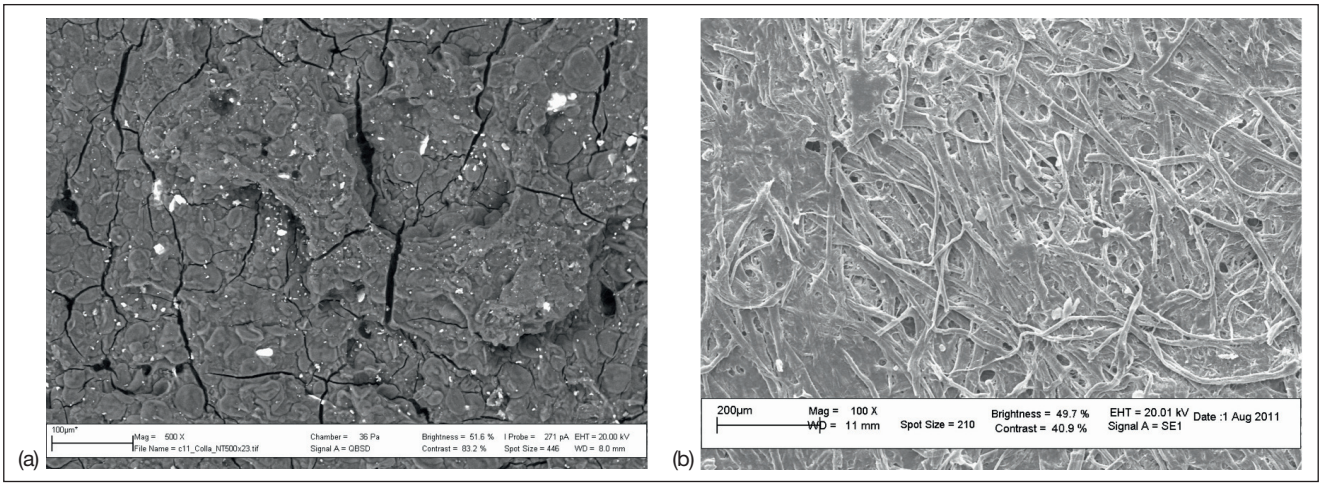

FIGURE 4 Images (SEM 500x) of a paper specimen before treatment, where the glue layer is evident (a), and after 4-hour treatment with the strain TSNRS 15 immobilised in agar gel, where the cellulose fibres are disclosed (b)

Source: Doctoral thesis Barbabietola, 2012. SEM analysis was kindly performed by ICRPAL laboratory

observations, performed after 4 hours of treatment, showed cellulose fibres appearing from the compact paste layer, demonstrating the disappearance of the adhesive layer consumed by bacteria as a carbon source (Fig. 4). Conversely, the observation of the samples treated with the sole agar gel, bacteria-free, shows significant glue residues, characterized by globular clusters, clearly visible on the surface. The final stage of the procedure is designed to check out that no undesirable residues are left over after the treatment, in order to avoid both the continuation of undesired metabolic processes and possible secondary colonisation due to the remains of the organic gel carrier. Actually, both streaking a swab after the treatment and SEM observation demonstrated that no residual bacteria or gel fragments were detectable.
The advantage of the developed procedure, based on living bacteria, over the current methods of glue removal, based on enzymes, lies in the metabolic versatility of microorganisms, that can adjust their action according to the changing conditions.

Partnerships

In partnership with the University of Florence two doctoral theses, awarded by the University, have been carried out at ENEA leading to scientific knowledge on shellac biodegradation; establishing a bio-cleaning procedure of original specimens of ancient paper kindly provided by the National Institute for Graphics, and exploring the application of biomolecules with surfactant properties (SACs), produced by selected microbial strains, for the removal of deposits or coatings from artworks. On the latter topic cooperation with the Department of Biomedical Sciences of the University of Cagliari is in progress.
With the Biology laboratory of the Central Institute for Restoration of the Book Heritage (ICRPAL), collaboration is underway on a diagnostic investigation of a biodeteriorated ancient parchment. Recently, collaboration has been extended to the University of Arizona.
A multidisciplinary study of an ancient Roman fresco fragments from the archaeological site Casa di Augusto at the Palatino is underway, in collaboration with the Special Superintendency for Archaeological Heritage of Rome, the Architectural Conservation and Restoration of University of Lund (Sweden), the Chemical Engineering Department of the University of Rome "Sapienza" and the Applied Physics Institute-National Research Council, Florence.
In collaboration with Small Restoration Enterprises, operating with different Institutions and especially with the Special Superintendencies for Archaeological Heritage of Rome and of Southern Etruria, preliminary trials are successfully in progress on frescoes in the Lodge of Casina Farnese, at the Palatino site, for the removal of hardened layers of casein and deposits from urban pollution.
Regarding activities on stone bioconsolidation, we thank the laboratory of magnetic resonance "Annalaura Segre", of CNR of Rome 1, for the kind willingness to cooperate with us.

Concluding Remarks and Perspectives

Biotechnology applied to restoration is an area still under development. In perspective, optimal restoration solutions require strong interaction among bio-based methods and physical- and chemical-based methods, as well as a better understanding and dialogue between the scientific-technological world and the art-restorers world.
Within this framework the research presented in this paper has led to advancement in the state of the art, both in terms of knowledge and potential applications. The research on shellac biodegradation represents the first attempt for its removal through a biological approach and has provided knowledge to develop a microbial formula for the removal of shellac without using toxic products. A successful procedure for the bioremoval of glues from ancient paper has been established, using for the first time immobilised living bacteria, instead of enzymes, expensive and of difficult application. All the bacteria used in this research have been tested for the first time for these purposes and have been specifically selected among numerous environmental strains of the ENEA laboratory collection, isolated and characterised for biotechnological application. All strains r-DNA 16S sequences are deposited at the GeneBank.

Thanks to the skilled partnerships, scientists as well as conservationists, we have the opportunity of expanding and deepening our investigations and the chance of testing our original products (microorganisms and bio-molecules arising from them) on real specimens. In perspective, this point is of great help to develop specific procedures for bio-restoration.

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