Biorestoration, exploiting the metabolic capabilities of microorganisms and their derivatives, is an effective support or a valid alternative to traditional techniques in the field of Cultural Heritage Conservation and Restoration.

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Search for new Surface Active Compounds of microbial origin in view of the development of biorestoration techniques

Faculty of Mathematical, Physical and Natural Sciences

PhD in Science for Conservation of Cultural Heritage
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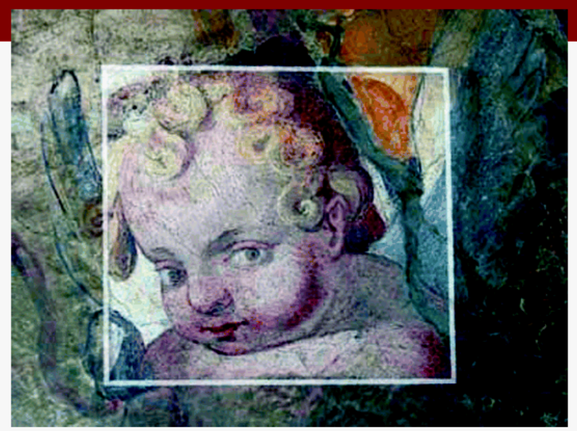

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A zio Cesare
che a suo modo mi ha
sempre incoraggiato
e continuerà a farlo

Cover image: A detail of the loggia's fresco of the Casina Farnese, in the archaeological site of the Palatine Hill, Rome (http://www.wmf.org/project/casina-farnese-palatine-hill).

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1. CHAPTER
INTRODUCTION

Europe's Cultural Heritage is the world's most diverse and rich patrimony attracting millions of visitors every year thanks to monuments, historical city centres, arhaeological sites and museums. Moreover, this heritage is an important component of individual and collective identity. In both its tangible and intangible forms[1], it contributes to the cohesion of the European Union and plays a fundamental role in European integration by creating links between citizens.

European Cultural Heritage has an exceptional economic importance for the tourism industry, generating an estimated annual revenue of 335 € billion[2], and many of the 9 million jobs in the tourism sector are linked to it directly or indirectly. The market for

[1] For the definition of "tangible and intangible cultural heritage", refer to: UNESCO, Convention concerning the Protection of the World Cultural and Natural Heritage, Paris, 16 November 1972; UNESCO, Convention for the Safeguarding of the intangible Cultural Heritage, Paris, 17 October 2003.

[2] The Economy of Culture in Europe, a study carried out by KEA European Affairs for the European Commission, 2006, pp. 147-155 and pp. 303-306.

conservation of this heritage is estimated around EUR 5 billion per year.

But this huge and invaluable patrimony is fragile, and it is estimated that in the past Europe has lost a great part of it, not only as a result of natural disasters, wars, negligence, vandalism and even terrorism, but also because of accelerating global change in its most general sense.

Pollution, urbanisation, deforestation, over-exploitation of water resources and other environmental changes, all affect the European patrimony. Coastal erosion and increasing human activity connected to the sea , threaten prehistoric and historic coastal sites submerged by a sea level that has been rising since the end of the last ice age. Mass tourism brings undoubted benefits but increases the risk of further degradation and of malicious acts. In the recent past, climate change is becoming a very important damage factor, adding to the other risk factors and contributing greatly to the deterioration of cultural assets.

Protection of cultural heritage in the face of global change is thus becoming a major concern for decision-makers, stakeholders and citizens in Europe. The necessity of safeguarding moveable and immoveable cultural heritage of European significance was only recognized by the EU Treaty of 1993 which specified that this area must be handle as a priority for the EU.

The Cultural Heritage represents an immense wealth for the whole human race, source of inspiration and reflection, a sign of talent, tenacity, passion and creativity of our ancestors, who crossed the narrow confines of time than a human lifetime. In this European *scenario* fits Italy: according to UNESCO, in our country are located 2/3 of the world's artistic heritage.

Their preservation, our responsibility, is severely tested by the constant attacks to which they are subjected.

In fact, from the moment the work of art is produced and included in the "biosphere", a slow but inexorable aging process begins. According to the second principle of thermodynamics in any "system" there is a general trend of increasing entropy. The Cultural Heritage, subjected to a daily assault of physical, chemical and biological, not exempt from this law. The environmental parameters can, , encourage their biotransformation, changing structure and composition, which reaches its highest levels in areas with hot and humid climate. This perennial struggle leads to a continuous shift in the balance, allocating the artwork to a fatal destruction (Magaudda 1994).

The aim of conservation action is to reduce the speed of deterioration process, being unable to totally stop.

Fundamental is a prior knowledge of the artwork composition, environment of conservation and factors responsible for the deterioration.

The study of the interactions between the biological world and works of cultural interest are the fundamental subjects of the biology applied to Cultural Heritage.

Both indoor and outdoor artworks continuously interact with biological world. This continuous contact generally has negative consequences such as the compromising of the state of conservation of the artistic work (Magaudda 1994).

In this perspective, biodeterioration defines the set of physical and chemical processes inducted by the growth of organisms that, transported by air or water, are deposited on the artistic surfaces causing the alteration.

The biodeterioration entails both damage of physical type, with disintegration of substrate caused by the penetration and growth of biological structures in the substrate; both damage of chemical type, which, causing a transformation of the chemical nature of the substrate, are much more complex and widely diffused than to those of a physical nature. Usually, they occur simultaneously, but according to the agents of biodeterioration, the type of substrate and the environmental conditions, may prevail one or the others. These actions may be as a result of the using of the artwork , not only as a food source by deteriogen organisms, but also as a physical medium for their growth (Tiano 1998).

The biodeterioration can be manifested in different ways, distinguishable as follows:

- *Removal of substrate*. The organisms, which at various levels and in different ways feed at the expense of the cultural property, will impoverish both organic and inorganic components and, altering the structure and mechanical properties, they can also destroy it.
- *Production of aggressive substances*. During their metabolic cycle, many organisms secrete substances such as acids, alkalis, chelating agents, which can corrode the constituent material of the work
- *Mechanical effects.* Typically they are caused by organisms of larger dimensions, such as plants (with their root systems) and animals.
- *Simple presence of the organism on the surface of the artwork.* It is an aesthetic damage, related to readability, and therefore to usability of the cultural heritage.

Furthermore, the development of micro-organisms or organisms can create favorable conditions to the growth of other species of biodeteriogens, or give rise to an ecological succession with the development of more complex and potentially harmful biocoenosis.

Depending on the different chemical composition of the materials, we can distinguish different types of biodeterioration. Works of art made from organic materials (paper, wood, textiles,

leather, etc..) are attacked especially by heterotrophic microorganisms capable of enzymatic degrade of organic substances. The inorganic materials are more easily attacked by autotrophic microorganisms, usually supported by heterotrophs.

The term *biodeteriogens* refers to a wide variety of organisms (and microorganisms) with extremely diversified modes of attack. We mention birds, rodents (among animals), insects, algae, fungi, lichens, and bacteria. Among living organisms which arouse major concerns for the health of the Cultural Heritage, the microorganisms occupy a prominent place. The study of microbiology related to the problems of Cultural Heritage conservation is a very interesting and promising field. The focus is, in the first place, on the identification of microorganisms sampled on works of art, the role played in the biodeterioration, the attack modes and finally the new prevention and recovery methods.

1.1 The role of microbial biotechnologies in the field of Cultural Heritage

Today, science and in particular biological sciences, have an important role in protecting and conserving Cultural Heritage for future generations (Rinaldi 2006). This task represents a great challenge - given the magnitude and diversity of objects involved,

and the enormous variety of different materials used (stone, metal, ceramics, paper, synthetic substances and organic matter) – not only for conservators, art historians and restorers, but also for researchers, scientists and politicians. At European level, the policy has sought to identify the best, most sustainable means of preserving our art. The basic principles of each restoration work, as defined in 1964 by the Venice Charter, are those of minimum intervention, reversibility, repeatability and retreatability. The development of science and technology have brought profound changes in human perception of the world and the environment resulting, therefore, also an evolution in the conservation principles.

In the last fifty years, it has gone from an extreme confidence in human capabilities and unlimited progress, typical of nineteenth-century vision, to the knowledge of the limits of man and his inability to control the world. A new perspective, aware of the complexity of life, both culturally and ecologically, was consolidated. All these changes have led to a reinterpretation of the principles of conservation and restoration. Thus the principle of minimum intervention, aware of the life-cycle of all materials, was developed in the more modern principle of "acceptable levels of damage". The principles of reversibility and repeatability have been replaced by that of compatibility and retreatability. 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, while preventive conservation is closely associated with environmental sustainability.

These theoretical changes in the field of conservation have led to changes in the scientific and technological approach to Cultural Heritage. In recent decades, science has played an increasingly important role in the study, restoration, conservation and valorization of Cultural Heritage. Today, the landscape is dominated by biotechnology and applied microbiology. These methods have allowed us to improve the diagnostic techniques for the identification of biological agents (very often organized in microbial communities) and their bio-deterioration potentialities towards the different materials constituting the artworks (Fernandes 2006). The knowledge of the metabolic capacity of the biodeteriogens not only has favored the development of techniques for the prevention of future colonization but also, more recently, has been exploited for the bioremediation of deteriorated artworks. Several studies show the microorganisms as a potential resource for biotechnological applications for cleaning (Saiz-Jimenez 1997) and consolidation of Cultural Heritage (Palla *et al*. 2006).

"Biotechnology - as defined by The Convention on Biological Diversity - United Nations of 1992 - *is the*

technological application that uses biological systems, living organisms, or their derivatives to make or modify products or processes for a specific purpose", that is the practical use of biological knowledge in order to obtain goods or services.

Over the last three decades biotechnology has played a vital role in agriculture, medicine, chemical and pharmaceutical industries, in the food sector and the environment. These technologies, although much discussed when involving the use of GMO (genetically modified organisms), present interesting advantages, compared with traditional techniques, such as low environmental impact, the absence of risks for humans, very low cost (compared to chemical-physical treatments) and low energy consumption.

Only recently, however, these technologies have found a place in the field of conservation and protection of Cultural Heritage. The introduction of molecular techniques , as already seen, has increased the number of microorganisms that can be studied, and in particular has enabled a deepening of their role in the process of deterioration of the various materials, such as stone materials, metallic or organic nature. This new approach has also highlighted the possibility of exploiting the microbial and enzymatic activity with an effect of "bioremediation" of deteriorated materials of cultural interest (Fernandes 2006).

Methods of molecular analysis have played a key role in reaching a better understanding of the micro-organisms and, in particular, the dynamics of colonization that succeeds on the substrates, in addition to the traditional cultural techniques and microscopic observations. The limit of the traditional approach is that only a very small part, it is estimated about 1%, of the members of microbial communities can be cultivated in the laboratory. As a consequence, our knowledge of the processes and microbial species involved in specific deterioration events has been seriously limited.

New molecular techniques based on the extraction, amplification and sequencing of microbial DNA , offer a way to fill the existing gap between the detection of cultured and uncultured microorganisms (Cutler e*t al*. 2010).

The DNA extracted may then be amplified via a polymerase chain reaction (PCR), using appropriate primers to select the organisms of interest (Stiller & McClanahan 2005). Cloning and amplification by PCR permits the use of techniques such as terminal restriction fragment length polymorphism (TRFLP), denaturing gradient gel electrophoresis (DGGE), fast in situ hybridization (FISH) and DNA sequencing.

Molecular methods allow to significantly reduce the quantity of sample to be taken, with a huge advantage for the integrity of the artwork; in fact, micro-sampling permits to obtain complete and specific framework of microbial communities present on the artwork,

with a phylogenetic study of sequences (Schabereiter-Gurtner *et al.* 2001), to identify and characterize the species already known and unknown (Palla & Tartamella 2006). The combination of PCR amplification of bacterial 16S rRNA genes (16S rDNA), phylogenetic sequence analyses, and genetic community fingerprinting by DGGE has been shown to be a proper method to describe species able to survive in extreme conditions, such as oligotrophy, exposure to high UV radiation, low water disposition and high saline concentration (Urzì 1999). A surprising example is the study of the deterioration of the mural paintings found in Saint-Catherine Chapel, Herberstein Castle (Austria), where the molecular analysis showed the presence of members of the halophile group of Archaea, *Halococcus morrhuae*, considered inhabitants of hostile environments, never found in historic and artistic contexts. (Schabereiter-Gurtner *et al.* 2001).

The use of molecular techniques also fits perfectly in a prevention policy, which, both from a theoretical as economic points of view, is the best "intervention" of Cultural Heritage. Molecular techniques make it possible to detect early and, then, to stop the possible biodeterioration process of a substrate, even before the degradation of the same is evident and the damage is irreversible. Similarly, they become a very useful tool for monitoring the efficacy of a treatment for restoration in the short and long term. The molecular studies allow, in fact, to evaluate with "micro-destructive"

samples the success of a restoration and conservation of works of art, such as the application of biocides.

At present, these new techniques represent an effective complement (not a substitute) to more classical microbiological methods. The fusion of these strategies is likely to result in a much better understanding of the number, activity and function of microorganisms, as well as revealing the importance of the identified microorganisms, not only in the bio-deterioration process, but also for their positive function in restoration and conservation.

1.1.1 *Bio-restoration*: a new biotechnological approach for the conservation and restoration of Cultural Heritage

The new biotechnological approach in the context of Cultural Heritage has shown that it is possible to convert potentially negative factors for the artwork in interesting factors for a new conservation optical. Exploiting the knowledge of the specific metabolic activities, you can use the *destructive* action of microorganisms for the biological removal of surface coatings on artwork, or even convert it in a *constructive* action, able to induce the precipitation of calcium carbonate deposits on carbonatic materials.

The study of the positive and constructive interactions that can be established between microorganisms and artistic substrates is called *Bio-restoration*.

The biocleaning of frescoes (Ranalli *et al.* 2005), the removal of black crusts and salts from stone (Heselmeyer *et al*. 1991, Ranalli *et al.* 1997; Cappitelli *et al.* 2007), the bio-consolidation of carbonatic materials (Castanier *et al.* 1999; Tiano *et al.* 1999; Bang *et al*. 2001; Rodriguez-Navarro *et al.* 2003, Barabesi *e al.* 2006; Sprocati *et al.* 2007) represent examples of where microbial technology may contribute positively.

The importance of new technology for Cultural Heritage is evidenced by the development in the last 20 years, of several projects in Europe, with over two hundred partnership and a multidisciplinary approach, aimed at the preservation of Cultural Heritage. In this new perspective fit European projects, in the Fifth Framework Programme (http://ec.europa.eu/research/). I will mention just a few:

- **BIOBRUSH (**BIOremediation for Building Restoration of Urban Stone Heritage), created to investigate how biotechnology can be used for the conservation of stone monuments and frescoes deteriorated due to pollution (2002-2005).

- **BIODAM,** a multidisciplinary project created to identify and inhibit the damage caused by biofilms on stone surfaces exposed to environmental hazards and biogenic (2002-2005).

- **BIOREINFORCE** (BIOremediated calcite precipitation for monumental REINFORCEment), project designed to develop a new consolidation method for stone materials based on the precipitation activity of some microorganisms (2001-2004).

- **PAPYLUM**, the aim of which was to study the phenomena of chemiluminescence during the degradation of the paper, through the construction of a new tool that allows non-destructive measures for the sample (2001-2004)

- **SYDDARTA** (SYstem for Digitization and Diagnosis in ART Applications**).** The main target is to develop a pre-industrial prototype for diagnosing the deterioration on movable assets by the acquisition of 3D-hyperspectral imaging through scanning non-destructive techniques (2011-2014).

- **JHEP**, Coordination action in support of the implementation of a Joint Programming Initiative (JPI) on Cultural Heritage and Global Change : a new challenge for Europe. A coordinating action which is intended to structure and support the new Joint Programming Initiative in Cultural Heritage and Global Change. The project aims to help with the development of a new Strategic Research Agenda for cultural heritage research (2011-2014).

One of the main interesting aspects of these new methods is that the microbes involved in the bio-restoration processes have been

selected among the native microbial populations inhabiting historical-artistic handworks, with enormous advantages compared to 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 *et al.* 2006).

These new bio-restoration solutions mainly relate to two kinds of intervention: bio-consolidation, by which organisms and microorganisms involved in the mineralization process, are used for the consolidation of deteriorated stone monuments and bio-cleaning, which employs viable bacterial cells or their derivatives to remove altered organic or inorganic patinas from artworks.

1.1.1.1 Bio-consolidation

An interesting application of bacterial cells in the field of Cultural Heritage is the work of Tiano and Mastromei (1999). It deals with microorganisms capable of precipitating calcite on calcareous materials, in order to achieve a re-calcification of degraded substrates.

The stone materials, especially for the works exposed to air, are continuously under the aggression of atmospheric agents, but also of factors of anthropogenic nature (industrial activities, urbanization, transport and domestic heating), which accelerate the

natural process of deterioration. One of the major causes of degradation of calcareous materials is the increase in porosity, resulting in a decrease in cohesion within the substrate and induction of an irreversible dissolution of the mineral matrix. This progressive loss of material produces a weakening of the substrate, making it necessary operations of consolidation, in order to increase cohesion between the various constituents of the article. The problems associated with traditional treatments of consolidation, such as incompatibility between the stone and the material applied, low penetration, scant solubility and high degradation, in particular for organic products, have prompted to development of this new biotechnological approach, based on the induction of calcite crystals inside the pores of the stony material; this process, a new and interdisciplinary research area, is called bio-mineralization (Ehrlich, 2000).

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 of a European project BIOREINFORCE (http://www.ub.es/rpat/bioreinforce/bioreinforce.htm).

The first application (Tiano 2005) was based on the isolation of macromolecules of the Organic Matrix Macromolecules (OMMs), extracted from the shells of the mollusc, *Mytilus californianus*, and other organisms that control the processes of formation of calcareous

structures, such skeletons, shells or teeth. The process of formation of calcite crystals, supported by the aspartate-rich macromolecules, thanks to their special arrangement facilitate the capture of calcium cations, has been studied and recreated *in vitro*. Studies of the crystals produced in mollusc shells show often the presence of aragonite minerals, on rare occasions calcite is present; only in few *taxa*, the same shell can have aragonite and calcite layers together (Wheeler and Sikes 1989).

Due to the difficulty of extraction of OMMs and long and expensive production times, despite the good results obtained on small limestone samples (Tiano 1998), this method is rarely appropriate for purposes of bio-consolidation. For these reasons, the attention has been directed to calcinogenic microorganisms.

With the project developed by "European Art Restores" (http://www.ub.es/rpat/bioreinforce/bioreinforce.htm), the ability to precipitate $CaCO_3$ on the surface of monuments, made of limestone by calcinogenic and non-pathogenic bacteria, was investigated, in order to exploit this process for innovative applications in preservation of stony monumental.

The formation of bacterial carbonates follows both autotrophic pathways (Castanier *et al.* 1999), which leads to an impoverishment of the local means of CO_2, and heterotrophic, which may be active or passive. The formation of $CaCO_3$ passive leads to an increase of pH and the production of carbonate and bicarbonate.

The carbonatogenesis active leads to the formation of carbonate particles by ion exchange, through the cell membrane and follows the mechanisms not entirely known (Cacchio et al. 2003).

However ,Bacteria do not have only an indirect role in the formation of calcite, with the variation of environmental parameters due to their metabolic activity, but also play a specific role. Thanks to the presence of reactive sites present on the cell surface, which facilitate the adsorption of metal ions dissolved, promote the nucleation and precipitation of neo-calcite.

Minerals formed by microorganisms can have a great variability of shapes, dimensions and colours (Sprocati *et al*. 2008) depending both on the microbial species and the environmental conditions in which the crystal precipitation occurs. A single species can in fact produce different crystals depending on the conditions in which it grows (Rivadeneyra *et al*.1999).

The first field experimentation was carried out on an area of 50 cm^2 in a S/E Tower of the Church of Saint Médard in Thouars (French) by the group of Le Metayer- Levrel G., Castanier S. and Orial. The treatment was performed in 1993 with an application (by spraying) of a suspension of *Bacillus cereus*.

On the basis of measurements taken in 1997 which confirmed the good quality and consistency of *biocalcin* and conservation of the outside of the tower (no colour alteration and reduction of water permeability), there were two patents (European

Patent nº 90400G97.0, Adolphe et al. 1990; French Patent nº 9505861, Castanier *et al.* 1995) and started the process CALCITE (Gonzales-Munoz 2008). In the CALCITE process they use the strain *Bacillus cereus*, which leads to the production of superficial protective coatings, called *biocalcin*, made up of calcified bacteria and calcified excretions, a few micrometers thick.

Numerous bacteria have been proposed as bioconsolidating agents. Rodriguez-Navarro C. *et al*. (2003), Jroundi *et al*. (2010) proposed the use of *Myxococcus xanthus*, a non-sporogenic gram-negative bacterium; Sprocati et al. (2008) selected a strain of *Rhodoccoccus erytropolis* among 71 calcinogenic strains isolated from the walls of an estruscan hypogeum (Fig. 1.2), while Jroundi *et al*. (2012), exploiting the natural community of altered stones, proposed *Acinetobacter* species as powerful carbonatogenic bacteria for calcareous stone conservation/consolidation.

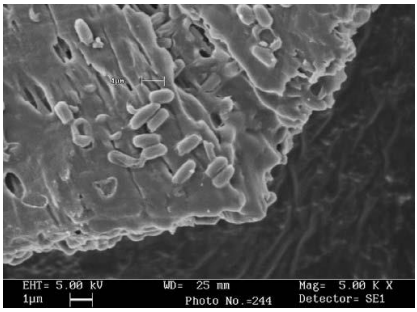


Fig. 1.1 Cells of Bacillus subtilis on the surface of a neoformation crystal (Tiano et al., 2005)

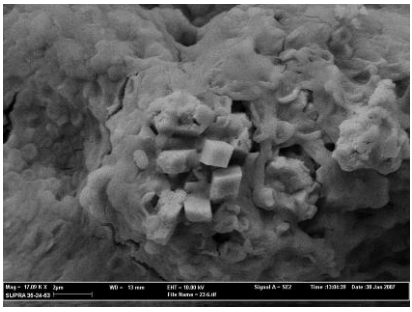


Fig. 1.2 Calcite crystals immersed in the biofilm formed by Rhodoccoccus erythropolis, on Pietra di Lecce stone samples (Sprocati *et al*. 2008)

Recently, it has been developed a protocol that involves the use of viable bacterial cells of *Micrococcus sp*. and *Bacillus subtilis* (Fig.1.1). The tests conducted on small samples of *Pietra di Lecce* showed that the two bacterial progressively induced the formation of new crystals and they led to a reduction of about 60% of the water absorbed by the treated samples. However, it is necessary to point out some problems that occurred during the experiment. First, the porosity of the stone is reduced thanks to the presence of large amounts of biological material, which physically obstruct the pores, and not solely due to the formation of neo-crystals. In addition, it was noted the presence of black and red spots, due to the growth of airborne contaminants, a consequences of the presence of organic nutrients in the media used to grow the bio-calcifying bacteria (Tiano *et al*. 1999).

In order to exploit the positive effects of bio-mineralization and to avoid the negative consequences, Barabesi *et al.* (2006, 2007) proposed a process of calcite precipitation without using viable cells. Surveys were carried out following a double approach: the identification of a bacterial cell fraction (BCF) able to induce the $CaCO_3$ precipitation (Barabesi *et al.* 2006) and the identification of genetic mechanism responsible for bio-mineralization (Barabesi *et al*. 2007).

Based on the promising results of the laboratory tests, *in situ* experiments were carried out. The BCF of *B. subtilis* was employed

for the treatment of three different monuments: the Church of S. Maria in Angera (fig.1.3), the castle of Champs-sur-Marne (France) and Tuffeau (artificial rock) (fig. 1.4 - Tiano *et al.* 2005).

Fig. 1.3: – Church of Santa Maria in Angera: Facade of the church (left) and application of the spray solution of BCF (right).

Fig. 1.4: Castle of Champs-sur-Marne (France) (above): selected areas for the application of the different products on the northwest wall Stone Merì (A) and artificial wall in blocks tuffeau, north facing (B). (http://www.trivella.it/foto/Arkos_12pp50-6.pdf)

Preliminary results show that the ability of the fraction BCF to form new calcite depends on the nature of the stone itself and by its porosity.

Starting from this background, was conducted a thesis (Barbabietola 2005/2006 – Sprocati et al. 2008) at the microbiology laboratory of the *Environmental Characterization, Prevention and Recovery UNIT* of ENEA-Casaccia. From the Etruscan tomb of Mercareccia several bacterial strains capable of precipitating calcium carbonates (bio-mineralizers) were isolated, allowing the establishment of a collection of strains producing crystals of different

colours and size, that may be useful for applications to differently colour gradation stones, some of them were not so far been described for calcinogenic ability. A few strains have been chosen for a trial of bio-consolidation on the Pietra of Lecce, by *in vivo* application. The strain *Rhodococcus erytropolis*, 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. The precipitation system was tested in vitro by the application of cellular fractions (BCF) harvested in the exponential growth phase, showing that at least in part the bio-mineralization is not dependent on active metabolism.

These results are encouraging for this new methodology, but further trials, both on a laboratory scale and *in situ*, are necessary.

1.1.1.2 Bio-cleaning

Another interesting kind of the interventions within the biotechnologies applied to Cultural Heritage is that of *bio-cleaning.*

One of the first phases of restoration is cleaning, which allows the removal of undesired deposits on the surface of the artworks. As the materials to be removed are strongly adhered to the surface of the work, the cleaning should be selective, without causing irreversible damage to the original work (Cremonesi 2004).

Bio-cleaning seems to satisfy these rules of accuracy, thanks to the research in the field of microbiology and molecular biology, which allowed the selection and characterization of bacterial strains with specific metabolic activities. The bio-cleaning has been put into practice by following two different approaches: the application of viable bacterial strains and the use of molecules of biological origin, such as enzymes, chosen for their selective action towards specific substrates.

The starting point is the enormous metabolic potentialities of microorganisms to induce biochemical transformations in a very wide range of substrates, and using them directly as a source of nutrition, and by releasing proteins with catalytic activity (enzymes) in the surrounding environment. Enzymes, such as amylases, proteases and lipases, are able to catalyze the degradation of macromolecules insoluble in water, by reducing them into smaller fragments. Their ability to hydrolyze substrates such as animal glues, starch, casein, wax, oil, synthetic resins (Buttazzoni *et al.* 2001), makes them suitable for applications in the field of Cultural Heritage.

The first attempts of cleaning with the use of enzymes date back to 1970. The work of Wendelbo (1970) based on the use of trypsin for the detachment of book pages stuck with animal glue, was followed by other interesting works for the restoration of parchment (Segal & Cooper 1977), paintings (Wolbers 1990; Cremonesi 1999), sculptures (Bonomi 1994), mural paintings (Klein *et al.* 2002), paper

and engravings (Banik 2002; De la Chapelle A. 2003; Iannuccelli *et al*. 2004).

The outcome of enzymatic cleaning depends on several factors, such as the choice of the right enzyme, the operative conditions and the way of applications. There are several types of enzymes that can be employed according to the substrate on which to act. But due to the complex nature of the artworks, in the majority of the works is necessary to use mixtures of different enzymes to have satisfactory results. Secondly, one must evaluate the optimal operating conditions, temperature and pH, for the enzymes, which must be compatible with the material to be treated. Generally, enzymes used in restoration have an optimal temperature around 37°C, then we must assess whether it is possible to heat the artistic surface to be treated. Similarly, some enzymes have an optimal pH around 1, but it is not reasonable to use a similar condition on an artwork surface. So the choice will focus on enzymes working in a range between 4 and 9.

The difficulty in obtaining optimal operative conditions during application, the use of substances (additives and thickening agents in the buffer solutions) which can contaminate the surface (Bellucci & Cremonesi 1994) and the high costs involved make enzymatic cleaning a procedure not accessible to all restorers.

To overcome the drawbacks that an enzymatic application can have in the removal of weathered and insoluble compounds,

another approach to *bio-cleaning*, which involves the use of viable bacterial cells, has been recently developed.

Compared with enzymes, the viable bacteria cells application seems to be less sophisticated and more efficient, thanks to the wide versatility of the bacteria, which produce both constitutive and inducible enzymes that attack and degrade different types of molecules. The synthesis of inducible enzymes takes place only in the presence of a substrate, creating a regulatory effect. Used in this way microorganisms prove to be efficient in the degradation of many chemical linkages, and are more effective than just the use of a single enzyme that attacks only specific linkages (Ranalli *et al*. 2005).

The main precautions to keep in consideration regarding the application of viable bacterial cells consist in the use of safe microorganisms that are non pathogenic and non-spore forming, in order to assure the safety of the treatment both for the operators and for the integrity of the artworks, and moreover in order to obtain the correct and complete removal of residual biological activity.

The first examples of viable bacteria applications for *bio-cleaning* procedures were carried out on stone for the bioremediation of black crusts. They represent a product of air pollution, which is formed in humid environments and in areas protected from the rain, following the conversion from calcium carbonate (constituent of many stone monuments) into calcium sulphate (gypsum), which then

incorporates in mineral matrix polycyclic aromatic hydrocarbons and carbonaceous particles, responsible for the dark color.

The first successful application of anaerobic sulphate - reducing bacteria was reported by Atlas et al. (1995) and Gauri and Chowdhury (1988), who used *Desulfovibrio desulfuricans* for the removal of sulphates from black crusts. The same *D. vulgaris* was exploited by Heselmeyer et al. (1991) for the conversion of gypsum crusts into calcite. In the following years, the research in this field continued. Saiz-Jimenez (1997) reports of microorganisms (cyanobacteria and heterotrophic bacteria) frequently isolated from black crusts, which have shown an extreme versatility due to their ability to use polycyclic aromatic hydrocarbons as sole carbon source. The ability of microorganisms to transform the deposits of atmospheric pollutants and to use them for growth was also highlighted in another work (Ortega-Calvo & Saiz-Jimenez 1997), in which is analyzed $^{14}CO_2$ in the conversion of [9-^{14}C] phenanthrene by bacteria isolated from the facades of two cathedrals of Europe (Seville in Spain and Mechelen in Belgium) and capable of using phenanthrene as the sole source of carbon and energy.

Based on these researches, Ranalli *et al*. (1997) proved the possibility of using different strains of sulphate-reducing bacteria, such as *Desulfovibrio vulgaris* and *D. desulfuricans* and denitrifying bacteria, *Pseudomonas stutzeri* and *P. aeruginosa*, in order to optimise a strategy for the removal of sulphates and nitrates

respectively from artistic handcrafts. In the experiment carried out on marble samples (employing the Sepiolite as carrier), about 80% of the nitrates and sulphates were removed by the bacterial metabolism after 36 hours of application.

In agreement with previous work (Ranalli *et al*. 1997), a methodology for the biotechnological removal of black crusts has been developed, through the use of *Desulfovibrio vulgaris* (sulphate-reducing bacteria, SRB), avoiding any precipitation or unwanted byproduct. The formation of precipitation of black iron sulphide was avoided by eliminating any source of Fe and using a filter that allows only the passage to bacteria, blocking the deposition of other aggregates. The sepiolite, used in the previous work, has been replaced by Carbogel, which requires less time colonization of the medium, keeps the coloration of the substrate, allows a greater efficiency (with a removal of 98% of sulphates in 45h) and an easy removal of the bacterial cells at the end of treatment (Cappitelli *et al*. 2006).

Fig. 1.5: Imagine of a fragment of stone covered with a black crust before (left) and after (right) the application of D. vulgaris (Cappitelli *et al.* 2006)

Webster and May (2006), in their article, highlight the drawbacks of traditional methods of cleaning: changes of the substrate color, migration of salts; too aggressive action of solvents may give rise to excessive removal, at the expense of the original material. They express, instead, confidence in the removal mediated by microorganisms, able to convert the gypsum of black crusts in sacrificial layers and, at the same time, consolidants of calcite. The sulphate- reducers bacteria are, in more detail, able to dissociate the gypsum into ions SO_4^{2+}, which is further reduced by the bacteria, and Ca^{2+}, which reacts with the carbon dioxide forming new calcite, according to the following reaction:

$$6CaSO_4 + 4H_2O + 6CO_2 \rightarrow 6CaCO_3 + 4H_2S + 2S + 11O_2$$

In a later work (Cappitelli *et al*. 2007) two methods of cleaning of black crusts, formed on marble taken from the Milan

Cathedral (fig. 1.6) are compared: a chemical method, with 50 g of ammonium carbonate and 30 g of EDTA, and one biotechnological, with a suspension of sulphate-reducing bacteria, *D. vulgaris*, in Carbogel. After two applications of 24h, on vellum paper, then covered by a PVC film to reduce evaporation of water and improve the yield of bacteria, the observations with portable microscope, SEM and FTIR spectrum, show effective cleaning action sulphates, without affecting the "noble patina", the veiling, an integral part of the meaning of the work, under the incrustation. In addition, neo-formations of calcite were observed, due to conversion by bacteria of gypsum, as seen above, which recognizes a further consolidating action for the substrate. The observations with portable microscope performed on samples treated with the chemical method show, on the contrary, a cleaning incomplete and non-uniform with inconsistent color black-gray residues. The surface, as the SEM photos show, is characterized by widespread and unwanted presence of tabular crystals of gypsum and microfractures.

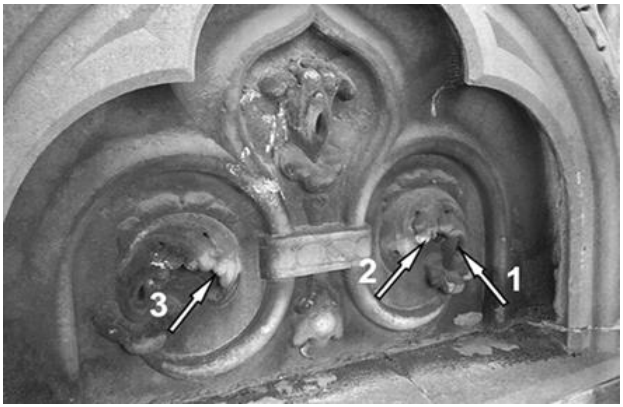


Fig. 1.6: Ornamental high relief frieze of Milan Cathedral chosen as the application site for the cleaning procedures. The arrows indicate the sampling areas (Cappitelli *et al.* 2007)

In recent years, a protocol (Ranalli *et al*. 2005), which envisaged the synergic combination of the hydrolytic enzymatic action with microbial metabolism, was described for the restoration of paintings. The occasion was offered by the restoration of one of the Pisa Cemetery's frescoes, "*Conversione di S. Efisio e Battaglia*", that, as a result of humidity and atmospheric pollution, was removed from the walls using the "tear-off" technique, i.e. by covering the surface with a strong cloth bound with generous layers of formaldehyde-treated glue (Antonioli *et al.* 2005). When, 20 years later, the curators attempted to remove the cloth, they found that the glue resisted any attack with surfactants and solubilising agents, even when treated with a mixture of the most aggressive proteolytic enzymes. The bioremediation method was the only way to restore the

fresco. It was performed by direct application of whole bacterial cells of *Pseudomonas stutzeri* A29 strain.

The operation was performed on different zones of the fresco, keeping the bacteria on the surface concerned, for a period of 10-12 hours at a temperature of 28-30 ° C (fig. 1.7).

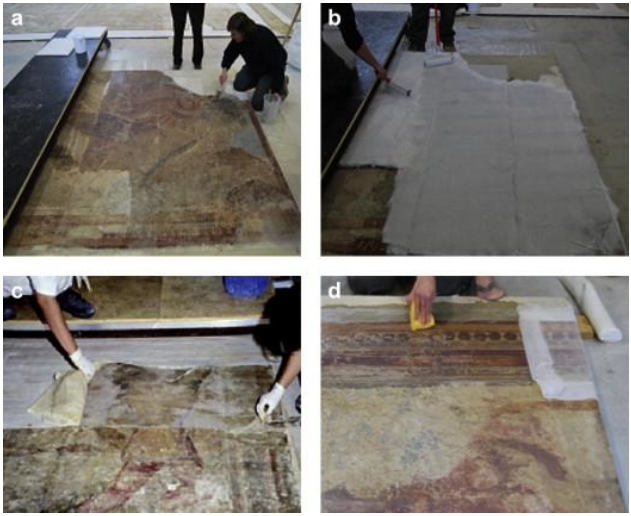


Fig. 1.7: Application (a-b) and removal (c-d) of packs of *Pseudomonas stutzeri* A29 on the surface of the "*Conversione of S.Efisio and Battaglia*" painting (Ranalli *et al*. 2005).

The biological pack removed 80-100% of the glue (fig. 1.8), leaving only small spot residues (never greater than *ca* 0,5 mm), which were eliminated with purified enzymes. In particular, after the first phase of degradation of most of the organic substance by

heterotrophic bacteria, enzymatic proteins, collagenase and protease, were used for the removal of residues. The protocol requires the bacteria to be removed completely at the end of the treatment from the painting , to avoid the possible triggering of deteriogen processes.

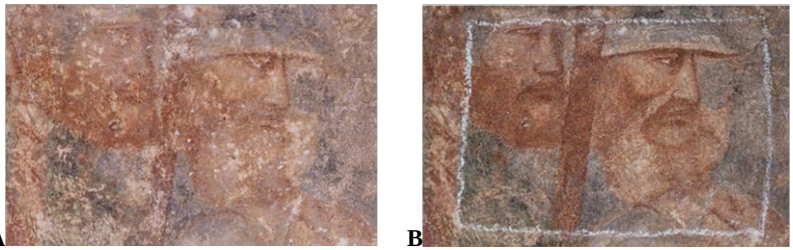


Fig. 1.8: A particular of the painting before (A) and after (B) the glue removal (Ranalli *et al*. 2005).

The characteristics of efficacy, absence of risk for delicate materials and not restrictive operating conditions, led us to develop in our laboratory a method of cleaning animal glue from aged paper. Original paper samples supplied by 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 *Ochrobactrum sp*. was selected because it was able to grow on both the 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 of the L* and ΔE* coordinates. SEM 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. 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 (Barbabietola N., PhD Thesis).

One of the most important advantages of biotechnological applications, over chemical treatments, is the absence of toxicity. Even now, all too frequently, toxic organic solvents, such as Toluol, Xylol, Dimethylformamide, Dichloromethane, are confidently used in the traditional cleaning methods of artworks (Bonaccini *et al.* 2001). On the contrary, the bioremediation treatments of the artworks are based on the use of aqueous media for the application of micro-organisms or their macromolecules. Water is one of the products most widely used in the field of restoration thanks to its

properties, the extreme versatility and economy, but, in certain cases, can become a risk factor. The uncontrolled spread of water in porous materials or surface alterations can cause damage to both physical-chemical and biological (Bellucci *et al*. 1994). Hence the control of water contribution in materials becomes necessary, in order to limit its diffusion or absorption on and under the surface. This can be achieved by adding specific substances, mineral or organic, which increase water viscosity. These substances can be distinguished in simple thickening agents, that are merely swollen up in water, such as Sepiolite or cellulose chemical pulp, or in gelling agents, which are completely dissolved in water such as cellulose ethers.

Other substances, used as a gelling agent in the field of Cultural Heritage conservation, were *Carbopol* (Bluher *et al.* 1996) agar and agarose (Brach *et al.* 2010), the Phytagel™, a synthetic polysaccharide made up of glucuronic acid, glucose and rhamnose, used in the cleaning of paper materials (Iannucelli *et al.* 2009).

Another interesting gelling agent, used in the restoration since the nineties, is Laponite RD. This gel, chosen for applications carried out within the present research work, has given excellent results even on very compromised, fragile and hygroscopic materials, such as paper, parchment paper and plaster models (Fornaciari 1993). The use of this "rigid" gel shows several advantages. It allows its simple removal after use, without leaving residues on the treated

surface and they make it easier applications even on uneven and non-flat surfaces, such as statues and mural paintings.

1.2 The surface cleaning phase in the restoration of Cultural Heritage

A work of art is usually formed by a complex sequence of layers (support, preparation, color layer, protective layer). Often on the surface of the image the presence of various materials occurs: products of natural alteration of the original painting, materials deposited over time, such as dirt, dust, fumes from various sources, and fat drippings patinas, or deliberately added in operations following the creation of the work, such as repainting, paint, materials for restoration (Cremonesi 1997).

Almost all the materials present on the surface of a pictorial image in time undergo structural alterations. These are often associated with chromatic alterations that can profoundly change the optical properties and thus the aesthetic appearance of the image, compromising the legibility and therefore the usability of the work. At this point, according to the concept of "restoration" as restoring the integrity of the work of art to allow usability, it is necessary to act with a cleaning operation on the surface of the artwork. This is one of the operations carried out more frequently in the restoration,

and one of the most complex for several reasons. It is an essentially irreversible intervention, because it tends to remove materials; in addition it takes place in a vital area of the work, the one in direct contact with the layer of color. Since the materials to be removed are located in direct contact with the surface of the work, the cleaning may cause irreversible damage so it must be performed in a selective way, through the removal of successive layers of deposit, differentiating the action between the area and the area, scantly or deeply removing without coming into direct contact with the original materials Among all the operations in the restoration, cleaning has the most immediate impact on the viewer. In this sense it should not be underestimated how important the factor "aesthetic" is: it brings a restoration to the taste of that particular historical period in which the action has taken place: over the centuries it has gone to the extremes, from the myth of preservation of the *noble patina* to the idea of eliminating it completely to restore the work to its original status. All these factors combine together to make the cleaning a very critical operation (Cremonesi 1997-2004).

The cleaning techniques have, therefore, suffered in the past and are still suffering continuously reviews with the aim of improving the performance of products and adapting methods of application to the recovered respect to the surfaces of these artworks.

1.2.1 The history of the surface cleaning and ethical controversies

Historically, in conservation and restoration treatises on artworks great attention has been paid to surface cleaning. Manuals on the conservation traditionally have included debates on dry methods of surface cleaning, including the use of dusting brushes and cloths, erasers and sponges. Foodstuffs, including fresh breadcrumbs, "cakes", potatoes, and anions have also mentioned (Mora et al. 1984). Famous is the case of Urbino, devoted servant of Michelangelo, who was paid to perform periodic cleanings of The Last Judgment and the frescoes in the Pauline Chapel, without any specific knowledge, using linen cloths, and then slices of white bread penny, sometimes wet with wine and vinegar. Same systems are used, as widely documented, even in subsequent decades (Bonasanti 2003).

Some older published instructions for surface cleaning of paintings prove to be quite extraordinary. Theodore De Mayerne's manuscript from the seventeenth century suggests: "Melt common carpenter's glue, which is quite thick, and pour it, melted over your picture, leave it after it has set... on your picture – then lift it off, all in one piece. This brings with it all the dirt. See if this can come off without damaging the piece (Caley 1990)."

Water and saliva, used for spit-cleaning, are perhaps the most common materials used for surface cleaning of paintings. The

addition of materials to water (whether 'de-ionized' or not) has predominantly been limited to the addition of alkalis. Ammonia is perhaps the most common alkali that has been added to water in the twentieth century to adjust the pH of the solution for cleaning of easel and wall paintings (Mora *et al.* 1984).

These intervention techniques became outdated with the emergence and spread, in the seventeenth century, of the positive value of the passing of time and of the traces left by it. We recall the suggestive definition of the *patina* offered by Baldinucci in his dictionary of 1680: "*voce usata dai pittori, e diconla altrimenti pelle, ed è quella universale scurità che il tempo fa comparire sopra le pitture, che anche talvolta le favorisce*" (Bonasanti 2003). This position is radicalized with a denial of any restoration, throughout the eighteenth century, when still restoration was intended as a form of art and the painters themselves performed the restoration interventions. Still in the middle of the nineteenth century, the denial of the superficial interventions had his followers as Pietro Selvatico, a painter-restorer and author of the book on the education of today's Italian historical painters. In contrast, we find Peter Edwards, author of a very interesting work on the restoration, where we can find typical concepts of modern restoration, such as the differential cleaning according to the shares of the work. After his work, the restoration begins to acquire an ethical shape: that is, the

restoration operation falls within a broadest context, leading to consequences and moral reasons (Bonasanti 2003).

At the end of the nineteenth century, advancements in the petrochemical industry led to the development of surfactants and detergents. With these new materials available, in the twentieth century the approach to cleaning chemistry became more sophisticated and commercial, patented cleaning products found applications in conservation. The addition of "soap" to water-based cleaning systems is sometimes mentioned in cleaning manuals from the twentieth century, although there is no mention of the specific types of soaps nor description of their chemical properties (Stavroudis et al. 2005).

Products currently used for the surface cleaning

At present, the cleaning phase is based essentially on two methods, the physical-mechanical and the chemical.

There is a wide range of mechanical methods of cleaning, nowadays less and less used because of their extreme aggressiveness and their lack of selectivity: tires, Wishab sponges, pens, fiber-glass, knives, or even aeromicro-abrasive are the mechanical techniques more fully described (Nordio 2011).

The chemical cleaning is based on the exploitation of the chemical characteristics of various products, that followed each other

over time. From the second half of the twentieth century, the use of organic solvents has become common practice and the reasons are various: effectiveness and rapidity of action, low cost and apparent simplicity of use are basic requirements. These positive aspects are contrasted by low selectivity towards the materials that are removed, toxicity for the operator and the environment. Moreover, the nature of organic solvents, either polar or alkaline or acid, thanks to the high rate of diffusion, does not guarantee a limited superficial action, putting at risk the underlying layers, the original materials and therefore the integrity of whole of artwork.

In the face of severe problems associated with the use of organic solvents, interest in alternative methods increased. In particular, much attention has been focused on the use of water-based systems. These include enzyme preparations, the "artificial saliva" and preparations containing surfactants (Cremonesi 1997).

The use of enzymes for surface cleaning of works of art, made available by the development of biotechnology in the field of conservation of Cultural Heritage, has been already widely discussed in a previous paragraph (see cap. 1.1.2.2). Limits in the use of this system are: the restrictive operating conditions (especially temperature and pH) incompatible with the conservative well-being of artwork; the difficulty for a total removal and the consequent permanence of potentially harmful residues for the work of art; last but not least, the cost factor. Greater enzymes purity undoubtedly

signifies greater specificity and activity, but the cost can be prohibitive, precluding any possibility of actual use (Cremonesi 1997).

Since time, we know the cleaning properties of saliva applied to the painted surface, but, apart from the difficulty of obtaining large quantities of saliva, a biological reason for not recommending its use is the possible presence of bacteria that could trigger biodeterioration phenomena on artworks. The active principles responsible for the detergent and emulsifier action of the natural saliva can be reformulated in artificial preparations simulating the action of saliva, without reproducing the defects. The action of the "artificial saliva", which must be kept constantly refrigerated because extremely thermolabile, is rather bland, so it is only effective if used as an intermediate washing, in the presence of already partially hydrolyzed material (Bellucci & Cremonesi 1994).

Cleaning controversies

The origin of the controversy on the issue of surface cleaning goes back to at least two thousand years ago, when there the disagreement on the benefits of cleaning a picture is well documented. In the first century AD Pliny the Elder recorded that a painting by Aristiedes of Thebes was ruined by its restoration.

Until the twentieth century, arguments were based on supposition and sometimes they had serious short and long-term effects for the people and paintings involved.

Separate cleaning controversies arose in England, France e Bavaria in the mid-nineteenth century. The English debate was filled by complaints from J. Morris Moore, the writer and art collector, about the cleaning practice of the National Gallery, claiming that paintings had been "completely flayed", and that "...fine rich glazings have been scoured off", "…such is the rage for destruction…".From then on, such language has found its way into the glossary of cleaning controversies. The French cleaning debate resulted in the promotion of Frederic Villot, Curator of Paintings at the Louvre, to a purely administrative role in order to remove him from contact with pictures. The Bavarian conflict, originating from published criticisms of the cleaning process at the Munich Pinakothek, led to the introduction of a solvent vapour treatment, the "Pettenkoffer method", which caused the long-term damage of paintings treated using this system.

The outcry over the cleaning in 1936-7 of Velasquez's "Philip IV of Spain" by the National Gallery (London) was closely followed by controversy surrounding the 1947's exhibition of cleaned pictures at the gallery. As a result of the latter, a *Committee of Confidential Inquiry into the Cleaning and Care of Pictures in the National Gallery* was formed headed by J.R.H. Weaver, President of

Trinity College. This was the first time that arguments were addressed beyond the polemic to a scientific examination of the cleaning products.

The early 1960s saw the development of a debate that involved major figures in the field of art history, conservation practice and conservation science. The improved understanding of solvent action made it possible to remove all accretions not applied by the artist. Despite this, the authors concluded that it was impossible to make the painting look as if it had just left the artist's easel, because of the changes occurred as the result of the painting aged. As the understanding of the physical nature of artworks grew, a sophistication in cleaning techniques evolved, including the concept of "patina". A general definition of "patina" is that it represents the desirable changes that occur over time (Khandekar 2001). So, conservation treatments are intended only to remove the "undesirable" or "vile" changes. However, the definition of "patina" is subject to changes and trends, giving rise to controversy. In the nineties the need to maintain the patina was strongly linked to the need for greater control in the process of cleaning (Beck *et al*. 1993).

There have been many more skirmishes over the cleaning of artworks surface, but the examples mentioned is usefull to show the professional and public concern regarding the cleaning of art work.

1.3 The surfactants and their use in the surface cleaning

The term "surfactants" was coined in 1950, as a contraction of "surface-active agent" (Rosen & Kunjappu 2012) to indicate substances that share a specific characteristic: a certain action "of surface" to the liquid in which they are dissolved. Even the term detergent is often used as a synonym because it highlights another property that all these substances, in appropriate concentrations, possess (Cremonesi 2003). Surfactants or SACs (= Surface Active-Compounds) are compounds that lower the surface tension of a liquid, the interfacial tension between two liquids, or between a liquid and a solid. Surfactants may act as detergents, wetting agents, emulsifiers, foaming agents and dispersants. Surfactants are usually organic compounds that are amphiphilic, meaning they contain both hydrophobic groups (their tails) and hydrophilic groups (their heads). Therefore, a surfactant contains both a water-insoluble (or oil soluble) component and a water-soluble component. Surfactants will diffuse in water and will adsorb at interfaces between air and water or between oil and water, when water is mixed with oil. The insoluble hydrophobic group may extend out of the bulk water phase into the air or into the oil phase, while the water-soluble head group remains in the water phase. This alignment of surfactants at the surface modifies the surface properties of water at the water/air or water/oil interface (Myers 2006).

In the bulk aqueous phase, surfactants form aggregates, such as micelles, where the hydrophobic tails represent the core of the aggregate and the hydrophilic heads are in contact with the surrounding liquid. Other types of aggregates such as spherical or cylindrical micelles or bi-layers can be formed. The shape of the aggregates is related to the chemical structure of the surfactants, depending on the balance of the sizes of the hydrophobic tail and hydrophilic head. This is known as the HLB, Hydrophilic-Lipophilic Balance. The formation of micelles occurs only after reaching a minimum amount of surfactant dissolved in water: this is called the Critical Micellar Concentration (CMC). The CMC, which can be influenced by various factors, such as temperature and the presence of salts, is characteristic of each surfactant. When the surfactant reached the CMC and the micelles were formed, the solution manifests emulsifying, detergent or solubilizer properties. The surfactants can be classified into Ionic and Non-Ionic, according to their hydrophilic part that can be or not be dissociated in aqueous solution, respectively.

Ionic Surfactants are subdivided into three groups. Anionic Surfactants are the ones ionized in aqueous solution, producing a negatively charged species, an anion. The most common are the carboxylates, sulphonates, sulphates and phosphates. Cationic Surfactants instead, are those ionized in aqueous solution, in the form of positive ion, a cation. Lecithin and benzalkonium salts are

two important examples. Amphoteric Surfactants are the ones ionized producing a species with both positions.

Higher is the number of Nonionic surfactants that, not giving rise to charged species in aqueous solution, exceed the limitations of ionic surfactants. (Cremonesi 2004)

The use of surfactants in restoration practice is recommended for several reasons: they impart particular properties, the so-called “surface properties”, to aqueous solutions or to organic solvents to which are added, and they possess a cleaning and emulsifier power.

The surfactants, if added in a small amount, increase the wetting power of the solutions, decrease the penetration of a solution through a porous body and the capillary rise (Cremonesi 1997). In the field of Cultural Heritage, these properties are commonly exploited to facilitate the sliding of a surface in an aqueous binder, to favor the contact between two incompatible surfaces (Wax / *colla di pasta*, Synthetic resins / *colla di pasta*), or to thin products such as lime mortar.

As regards the detergent and emulsifier activity of surfactants the importance for the purpose of surface artworks cleaning is evident. Nonionic Surfactants, such as Tween 20, Brij 35 and Triton X-100, are widely used in the restoration. They have been used for surface cleaning of the generic "dirt" with the fat component. However, a serious disadvantage of these surfactants,

especially Triton X-100, is that they are degraded forming pollutants by-products for environment and toxic for the operator.

The use of cationic and anionic surfactants in the restoration has instead remained d limited because of their acidic or alkaline character, respectively, which makes them not always compatible with the substrate. You can find commercially available many of these products, such as soaps or modern Resin Soap. Soaps are widely discouraged because they are characterized by an overly aggressive action against fragile substrates, such as artistic deteriorated surfaces (Cremonesi 2004).

In the 80's Richard Wolbers, restorer and researcher at the *Winterthur Museum* in Wilmington, Delaware, designed and developed two anionic surfactants, alkaline, generally called Resin Soaps (specifically the Resin Soap or ABA-TEA [abietic acid-triethanolamine] and Bile Acid Soap or DCA-TEA [deoxycholic acid-triethanolamine]). The action of Resin Soaps combines the wet and emulsifier ability to that dissociating and ionizing against the substrate. The structural similarity makes them particularly effective in the removal of even aged paints based on natural resins. There is not always a complete solubilization of the paint, so a subsequent action of a mechanical type or an aqueous solution of another surfactant is necessary.

Other two Soaps were subsequently developed at the National Gallery in London, by Raymond White from two different

compounds belonging to the class of polycyclic aromatic hydrocarbons. Despite their best structural interaction with the molecules of oxidized resin, they are highly toxic therefore not suitable as an alternative to Soaps of Wolbers.

The problem of Resin Soaps is that the original formulation involves the use of potentially hazardous surfactants, such as Triton X-100 that produces toxic by-products. In particular, the formation of peroxides can be a problem in the case of their permanence on the work of art, for the fact that these intermediates may catalyze the degradation of many organic compounds, and thus, accelerate the degradation of the work itself. This disadvantage can be overcome by using other surfactants. The problem of the assumed permanence of the residues still remains

Recent studies (Khandekar 2001) demonstrated that this problem remains even after a long time. Another controversial issue is the presence of Triethanolamine which, due to its non-volatility and strong hygroscopicity, results in a strong retention of residues in a work of art, with the risk of excessively soften and plasticize the underlying layers (Erhardt & Bischoff 1994).

The third way is to exploit the surfactants emulsifying property: mix together immiscible liquids, such as water and polar solvent. From the application's point of view, the emulsions are very important: combining the solvent power of the solvents with various properties of aqueous solutions can be useful for action on complex

layers of material (casein and oil, chalk with a synthetic binder, wax and resin) . Among the various types of emulsions, we can mention the Waxy Emulsion (said *Pappina Fiorentina*) used in the field of restoration not as clean-up agent but as a support of aqueous solutions. An example of application of particular importance is a microemulsion used to clean the frescoes by Masaccio in the Brancacci Chapel in Florence (Baglioni & Giorgi 2000). In the last decade, a novel class of "oil-in-water microemulsions" mainly based on nonionic surfactants was successfully tested in two real cases: a Renaissance painting by Vecchietta in Santa Maria della Scala, Siena, Italy, degraded by the presence of a polyacrylate coating applied during a previous restoration; and the *fresco* in the Oratorio di San Nicola al Ceppo in Florence, characterized by insoluble inorganic deposits strongly associated with asphaltenes, deposited by the water of Arno river during the 1966 flood (Carretti *et al*. 2007).

Particularly useful for specific interventions, such as cleaning of gilding leaf, are the so-called Fat Emulsions, composed of a small amount of water dispersed in a lipophilic solvent.

Other products based on surfactants used in cleaning are the Solvent Gels. Introduced by Richard Wolbers, these preparations are multi-component systems. Very briefly, they are surfactants in thickened aqueous solutions or organic solvents, in order to obtain a mixture of solvents of different polarities at a very high viscosity, with the following reduction of the spread into the substrate. The

major disadvantage in the Solvent Gels is the difficulty of complete removal of their residues from the substrate. In particular, the presence of Ethomeen, a non-volatile element, within the formulation, would result in its strong retention in the inner layers becoming a factor of degradation for the treated work. The quantitative analysis of gel residue using radioactively labelled gel components in combination with scintillation counting, conducted by Stulik *et al*. (2002), allowed to establish a direct relationship between the porosity of the material and the amount of gel residue, and confirmed that materials of higher porosity retain larger amounts of gel residue. These residues might also seal the pores of the cleaned material, changing its optical properties or the physical-chemical state of the surface, attracting and retaining dust particles.

The classification of the surfactants used in the restoration are summarized in table 1.1.

NON IONIC SURFACTANTS
- Contaminants and toxic by-products - Permanence of residues
IONIC SURFACTANTS
• ANIONIC
○ SOAPS
Aggressive action against fragile substrates
○ RESIN SOAPS
-Contains Triton X-100 that produce by-products which accelerate the degradation of the artefact - Contains TEA which, non-volatile and hygroscopic, causes softening and plasticizing of the underlying layers
• CATIONIC
Not always compatible for aggressive character toward the substrate
• AMPHOTERIC
EMULSIONS (Waxy, Fat and Micro-Emulsions)
Needed successive washings with solvents to remove residual
SOLVENT GELS (use of the surfactant as a thickener)
Permanence of residues

Table 1.1 Use of surfactants in the restoration of Cultural Heritage and main disadvantages

During the last few years, research has been directed towards cleaning techniques increasingly safe, selective, and therefore less invasive , able to exploit the full potential of the media thickeners.

A wide-ranging panorama of the surface cleaning techniques in the restoration of Cultural Heritage and of the various controversies arisen from the use of certain products testify the great technical, but especially ethical, importance linked to this fundamental step of the artwork conservation.

My research fits into this context, focusing on the biosurfactants, as a potential biological alternative to chemical-synthetic surfactants (and their derivatives), for the cleaning of artworks.

1.4 The Biosurfactants

Microbial surface-active compounds, generally called biosurfactants, are a structurally diverse group of surface- active molecules synthesized by different microorganisms and they are mainly classified by their chemical structure and their microbial origin (Desai & Banat 1997; Banat *et al*. 2010). Diverse ranges of prokaryotic and eukaryotic microorganisms are capable of producing surfactants (Christofi & Ivshina 2002).

The biosurfactants are amphiphilic molecules that have both hydrophilic and hydrophobic moieties, and therefore are able to reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures. The biosurfactants have different localization (e.g. intracellular, cell-surface bound and extracellular) and in nature they have divergent physiological purposes in the different production of microorganisms (Ruggeri *et al*. 2009). Although most biosurfactants are considered as secondary metabolites, some may play essential roles for the survival of biosurfactant-producing microorganisms by facilitating nutrient transport or microbe-host interactions or by acting as biocide and fungicide agents for the producing organism (Deziel *et al*. 1996; Jennings & Tanner 2000; Dehghan-Noudeh *et al*. 2005; Rodrigues *et al*. 2006a; Fernandes *et al*. 2007). Biosurfactant roles include: increasing the surface area and bioavailability of hydrophobic water-insoluble substrates (Al-Araji *et al*. 2007), heavy metals binding (Kavamura & Esposito 2010; Hoffiman *et al*. 2010), bacterial pathogenesis and biofilm formation (Fiechter 1992; Healy *et al*. 1996; Gautam & Tiagi, 2006; Simoes *et al*. 2010). Biosurfactants can interact with microbial proteins and can be manipulated in order to modify enzyme conformation in a way that alters enzyme activity, stability and/or specificity (Kamiya *et a*l. 2000).

Several studies have been conducted on the anti-viral properties of the Surfactin, a biosurfactant produced by *Bacillus*

subtilis (Vollenbroich *et al.* 1997), and on the anti-microbial properties (Benincasa *et al.* 2004) and permeabilizing effect (Satirova *et al*. 2008) of rhamnolipids produced by *Pseudomonas aeruginosa.*

Das *et al*. (2009) reported a biosurfactant produced by marine *Bacillus circulans* showing a strong antimicrobial activity against Gram-positive and Gram-negative pathogenic microbial strains.

Fernandes *et al*. (2007) investigated the antimicrobial activity of biosurfactants from *Bacillus subtilis* R14 against 29 bacterial strains. Results demonstrated that lipopeptides have a broad spectrum of action, including antimicrobial activity against microorganisms with multidrug-resistant profiles (Fernandes *et al*. 2007).

Rodrigues *et al*. (2006c) mentioned that MELs (mannosylerythritol lipids) produced by *Candida antartica*, rhamnolipids produced by *P. aeruginosa* and lipopeptides produced by *B. subtilis* 31 and *B. licheniformis* show antimicrobial activity (Rodrigues *et al*. 2006d).

Biosurfactants have been found to inhibit the adhesion of pathogenic organisms to solid surfaces or to infection sites (Das *et al.* 2009); thus, prior adhesion of biosurfactants to solid surfaces might constitute a new and effective means of contrasting colonization by pathogenic microorganisms (Rivardo *et al*. 2009). Furthermore,

Rodrigues *et al.* (2004) demonstrated that biosurfactants greatly reduced microbial load on prostheses and also induced a decrease in the airflow resistance that occurs on voice prostheses after biofilm formation.

These potential anti-microbial and anti-mycotic activities of several biosurfactant represent another interesting property that could be exploited in the Cultural Heritage field , in order to control and/or prevent biofilm formation (biodeterioration) on artistic surface.

Biosurfactants are also involved in promoting the swarming motility of microorganisms and they participate in cellular physiological processes of signalling and differentiation (Kearns & Losick 2003).

Microorganisms produce low and high molecular weight biosurfactants. The low molecular weight types are generally glycolipids or lipopeptides. The glycolipids include trehalose tetraesters and dicorynomycolates, fructose lipids, sophorolipids and rhamnolipids. Lipopeptides include surfactin, viscosin and polymixin. Glycolipids and lipopeptide substances are involved in the lowering of surface and interfacial tension of liquids. Stable emulsions are not a usual trait of these surfactants. High-molecular weight biosurfactants are amphiphilic (amphipathic) o polyphilic polymers which are usually more effective in stabilising emulsions of oil-in-water (Karanth et al. 1999; Rosenberg and Ron 1999) but do not lower the surface tension as much (Smyth *et al*. 2010a). Among

these, there are the polymeric biosurfactants, like as emulsan, liposan, mannoprotein and other polysaccharide-protein complexes, and the particulate biosurfactants, extracellular membrane vesicles which plays an important role in alkane uptake by microbial cells forming microemulsions (Monteiro *et al*. 2007; Mukherjee *et al*. 2006; Ortiz *et al*. 2006).

Thus, the majority of biosurfactants include low-molecular-weight glycolipids (GLs), lipopeptides (LPS), flavolipids (FLs), phospholipids and high-molecular-weight polymers such as lipoproteins, lipopolysaccharide-protein complexes and polysaccharide-protein-fatty acid complexes. Biosurfactants have a great deal of structural diversity. The common lipophilic moiety of a biosurfactant molecule is the hydrocarbon chain of a fatty acid, whereas the hydrophilic part is formed by ester or alcohol groups of neutral lipids, by the carboxylate group of fatty acids or amino acids (or peptides), organic acid in the case of flavolipids, or, in the case of glycolipids, by carbohydrates (Santa Annal *et al*. 2002; Ruiz-Garc *et al*. 2005; Rodrigues *et al*. 2006; Singh *et al*. 2007).

The best-studied microbial surfactants are glycolipids. Among these, the best-known compounds are rhamnolipids, trehalolipids, sophorolipids and mannosylerythritol lipids (MELs), which contain mono- or disaccharides, combined with long-chain aliphatic acids or hydroxyaliphatic acids. Rhamnolipid production by *Pseudomonas* species has been extensively studied, and potential

applications have been proposed (Maier & Soberòn-Chàvez 2000). Rhamnolipids from *Pseudomonas aeruginosa* are currently commercialized by Jeneil Biosurfactant, USA, mainly as fungicides for agricultural purposes or additives to enhance bioremediation activities.

Franzetti *et al*. (2008-2009) have conducted extensive studies on glycolipids products by *Gordonia* sp.

Sophorolipids, on the other hand, are produced mainly by yeasts, such as *Candida bombicola*, *Centrolene petrophilum*, *Candida apicola* and *Rhodotorula bogoriensis*, while MELs are produced by *Pseudozyma yeasts*, *Pseudozyma aphidis*, *Pseudozyma antarctica* and *Pseudozyma rugulosa* (Konishi *et al*. 2007a, b).

Cell-bound trehalose lipids are produced by *Rhodococcus* and other actinomycetes; a variety of structurally different lipopeptides are produced by several *Bacillus* species (Lang & Philp, 1998; Cubitto et al., 2004; Van Hamme et al., 2006).

In the last few decades, various bacterial species and various types of biosurfactants have been studied. In the table 1.1 the best known biosurfactants and the main proposed applications are listed.

Organism	Type of biosurfactant	Potential Applications	Reference
Rhodococcus erythropolis 3C-9	Glucolipid and a trehalose lipid	Oil spill cleanup operations	(Peng et al. 2007)
Pseudomonas aeruginosa S2	Rhamnolipid	Bioremediation of oil contaminated sites	(Chen et al. 2007)
Pseudozyma siamensis CBS 9960	Mannosylerythritol lipid	Promising yeast biosurfactant	(Morita et al. 2008a)
Pseudozyma graminicola CBS 10092	Mannosylerythritol Lipid	washing detergents	(Morita et al. 2008b)
Pseudomonas libanensis M9-3	Lipopeptide	Environmental and biomedical applications	(Saini et al. 2008)
Bacillus subtilis strain ZW-3	Lipopeptide	Potential in pharmaceutics, environmental protection, cosmetic, oil recovery	(Wang et al. 2008b)
Rhodococcus sp. TW53	Lipopeptide	Bioremediation of marine oil pollution.	(Peng et al. 2008)
Pseudozyma hubeiensis	Glycolipid	Bioremediation of marine oil pollution	(Fukuoka et al. 2008)

Organism	Type of biosurfactant	Potential Applications	Reference
R. wratislaviensis BN38	Glycolipid	Bioremediation applications	(Tuleva et al. 2008)
Bacillus subtilis BS5	Lipopeptide	Bioremediation of hydrocarbon- contaminated sites	(Abdel-Mawgoud et al. 2008)
Azotobacter chroococcum	Lipopeptide	Environmental applications.	(Thavasi et al. 2008b)
Pseudomonas aeruginosa BS20	Rhamnolipid	Bioremediation of hydrocarbon- contaminated sites.	(Abdel-Mawgoud et al. 2009)
Micrococcus luteus BN56	Trehalose tetraester	Bioremediation of oil-contaminated environments.	(Tuleva et al. 2009)
Bacillus subtilis HOB2	Lipopeptide	Enhanced oil recovery, bioremediation of soil and marine environments, and food industries.	(Haddad et al. 2009)
Pseudomonas aeruginosa UFPEDA 614	Rhamnolipid	Bioremediation.	(Neto et al. 2009)

Organism	Type of biosurfactant	Potential Applications	Reference
Nocardiopsis alba MSA10	Lipopeptide	Bioremediation	(Gandhimathi et al. 2009)
Pseudoxanthomonas sp. PNK-04	Rhamnolipid	Environmental applications.	(Nayak et al. 2009)
Pseudozyma parantarctica	Mannosylmannitol lipid,	Emulsifiers and/or washing detergents	(Morita et al. 2009)
Pseudomonas alcaligenes	Rhamnolipid	Bioremediation	(Oliveira, et al.2009)
Pseudomonas koreensis	Lipopeptide	Biocontrol Agent	(Hultberg et al. 2010)
Pseudomonas fluorescens BD5	Lipopeptide	Bioremediation and biomedicine.	(Janek et al. 2010)
Candida bombicola	Sophorolipids	Environmental applications.	(Daverey and Pakshirajan 2010a, b)
Brevibacterium aureumMSA13	Lipopeptide	Microbial Enhanced Oil Recovery (MEOR)	(Kiran et al. 2010b)

Organism	Type of biosurfactant	Potential Applications	Reference
Nocardiopsis lucentensis MSA04	Glycolipid	Bioremediation in the marine environment.	(Kiran, et al. 2010a)
Bacillus velezensis H3	Lipopeptide	Industrial strain for the Lipopeptide production.	(Liu et al. 2010)
Calyptogena soyoae	Mannosylerythritol lipid	Bioremediation processes in the marine environment.	(Konishi et al.2010)
Burkholderia plantari DSM 9509	Rhamnolipid	Detergents and pharmaceutical industry	(Hörmann, 2010)
Flavobacterium sp. MTN11	Flavolipids	Bioremediation of organic chemical- and metal-contaminated sites	(Bodour et al. 2004)

Table 1.1: Biosurfactants, producing organisms and their applications in recent years

Usually, different microbial genera synthesize structurally different classes of Surface Active Compounds (SACs) whereas strains belonging to a same species produce structurally similar SACs (Bodour *et al*. 2003). Several studies have shown that the

production of biosurfactants is affected by numerous environmental and nutritional factors, both qualitatively and quantitatively. The different carbon (Toledo *et al*. 2006) and nitrogen sources (Abouseoud *et al*. 2008), the pH, salts concentration, growth temperature (de Carvalho & de Fonseca 2005), culture time and aeration (Kyung-Taek Oh *et al.* 2006), influence the production of biosurfactants.

Surfactants offer extraordinary benefits to many industries. They are involved in infinite numbers of different industrial processes and physicochemical phenomenon; increasing mobility, increasing solubility, lubrication, removing soil or scouring (Pei *et al*. 2009; Lai *et al*. 2009), wetting, rewetting, softening, retarding dyeing rate, fixing dyes, making emulsions, stabilizing dispersions, coagulating suspended solids, making foams (Hirata *et al*. 2009), preventing foam formation and defoaming (Kosaric 1992; Zang and Miller 1992; Zouboulis *et al*. 2003).

In addition to all these benefits, biosurfactants have a large number of bioactivities, useful in the medical and pharmaceutical field: inhibition of bacterial growth (Flagas & Makris 2009; Sabate *et al*. 2009), stimulation of the immune system, tumor growth inhibition, antibiotic activity, cell lysis (haemolysis) (Dehghan-Noudeh *et al.* 2005), plant pathogenicity (Joshi *et al*. 2008), effects on migration of human neutrophils, action on the respiratory system (anti- asthma activity), food digestion (Nitschke & Costa 2007),

inhibition of cell wall synthesis, fungicidal properties (Joshi *et al*. 2008) or enzyme stimulation, bio-regulatory effects, etc. Hence they play part in many processes in nature (Rodrigues *et al*. 2006b).

These molecules have an unlimited number of uses that involves: oil industry, pharmaceuticals, testing quality of condoms, hygiene and cosmetics, cement, beer and beverages, textiles, paint, detergents and cleaning (Rai & Mukherjee 2010) and food processing (Arauz *et al*. 2009). However, the applications depend on the considered properties and the mechanism of action.

The most significant applications of biosurfactants are in the field of bioremediation (table 1.1), for example in removing heavy metals from soils (Franzetti *et al*. 2009; Gusiatin *et al*. 2009; Wang & Mulligan 2009; Mullingan 2009; Asci *et al*. 2010). Over recent years many studies have shown that biosurfactants can solubilize and mobilize organics adsorbed onto soil constituents (Zang and Miller 1992). Brusseau *et al*. (1995) used monorhabdolipid acid for the removal of residual NAPL (non-aqueous phase liquid), and, at a concentration of 500 mg l^{-1} , around 22% of residual hexadecane was removed from sand columns. Ivshina *et al*. (1998) used crude biosurfactant complexes from *Rhodococcus* strains (Mycolata taxon) to remove oil from sands and oil shales. Park *et al*. (1998) also examined surfactant production in a member of the Mycolata, *Nocardia erythropolis* (ATTC 4277), and showed micellar solubilization of hydrophobic substances. The percentage of crude

oil removed in the study by Ivshina *et al.* (1998) depended on the relative abundance of asphaltenes, resins and saturates in the crude oils used. Some strains produced surfactant complexes that removed almost 100% of oil sorbed onto sands, whereas the efficacy of removal for other strains was low (<10%) (Christofi & Ivshina 2002).

These and more recent successes in the use of biosurfactants in the environmental sector, and particularly in the bioremediation of water and contaminated soils (Banat *et al*. 2000; Haddadin Malik *et al.* 2009), where more than 100 polluting compounds can be removed by microbial surfactants, prompted us to investigate a possible use of biosurfactants in the field of restoration of Cultural Heritage.

The biosurfactants, thanks to the many surface properties, could provide an effective alternative of biological origin in the resolution of restoration issues (described above) in a delicate and controversial step of surface cleaning of artworks, by outlining a new approach to the conservation and restoration of Cultural Heritage.

Biosurfactants have several advantages over the chemical surfactants, such as absence of toxicity for the operator and the artwork, higher biodegradability (Lima *et al*. 2011), better environmental compatibility, high selectivity and specific activity at

extreme temperatures, pH and salinity and the ability to be synthesized from renewable feedstocks (Desai & Banat 1997).

This work, starting from these premises, represents a research of a new Surface Active Compounds (SACs) of microbial origin in order to develop a new biorestoration technique.

1.5 *Casina Farnese*: a case study

In order to establish a protocol for application of biosurfactants produced in our laboratory in real situations (the method will be described in the next chapter) and to ensure safety for the artwork and for operators, a cleaning test on a portion of the loggia frescoes of *Casina Farnese* have been performed, after the development of a cleaning protocol on lab-made specimens.

The Casina Farnese (fig.1.9) belonged to the greater Orti Farnesiani estate of the Farnese family during the 16th century. Small in size but architecturally and historically highly valued, Casina Farnese is one of the few Renaissance buildings that remains atop the Palatine Hill in Rome.

Fig. 1.9: Casina Farnese, in the archaeological site of the Palatine Hill, Rome.

Due to substantial archaeological excavations in the 19th and 20th centuries responsible for the alteration of the physical layout of the area, little is known about Casina Farnese before the changes with the exeption of some engravings, prints, and photographs. Remarkable figurative paintings and murals on the loggias, vault decorations, and the landscaping along the walls of the structure are its most appealing aspects. Its two-storied loggias facing the Tiber were exclusively designed by artists from the workshop of Taddeo Zuccari (1529-1566) and provide exquisite views of the city. The prized artwork and architecture of the Casina Farnese were long in danger of deterioration due to lack of maintenance and disrepair.

In 2007, WMF (World Monuments Fund, an American foundation whose mission is to preserve the world's architectural heritage of significant monuments, buildings and sites) began controversies with local authorities about the range of conservation challenges at the site, with particular concern for wall and ceiling paintings and damaged plaster surfaces of the two loggias. Water infiltration, clogged drains, wind, rain, and humidity caused the detachment of the murals and corrosion of other indoor decor. In conjunction with WMF, the Rome's Archaelogical Superintendency removed deposits of carbon and dirt from the surface of the paintings and conserved the deteriorating plaster and paintings. In 2008, cracks in the structure's surface and in the roof were repaired, and the drainage system was improved to prevent further leakage. During

this time, scaffolding was installed to protect the loggias. A survey showed that the overall instability of the building was caused by nearby archeological excavations, creating a large crack in the lower loggia leading to the breaking of the travertine cornice into many pieces. As a result, a simple support was installed. When the overall structure is stabilized, special glass panels will protect the paintings on the loggia. Long-term plans involve creating a small exhibition space for a collection of engravings, prints, and documents and the construction of a staircase to allow access.

According to the mith, the Palatine Hill was the location where Hercules defeated Cacus, a fire-breathing monster that had stolen a herd of cattle from Hercules while he was sleeping. The legend of Hercules and Cacus is painted on the vaults in each loggia of the Casina Farnese (fig. 1.10). The paintings enhance the story and represent the importance of Palatine Hill in Roman history.

As part of Casina Farnese redevelopment works, in 2012 the restoration of the frescoes in the loggias have been carried out by the restorers Adele Cecchini, Franco Adamo and their team (fig. 1.11) . The worse conservation status of the lower lodge has led to restorers several problems in the cleaning of the surface deposits of the frescoes. These deposits have been characterized, thanks to the work conducted by Dr. Maria Laura Santarelli of the Department of Chemical and Environmental Engineering Material of the Sapienza University of Rome, as mainly consisting of calcium caseinate,

partially degraded in complexes layers of lime phosphate, a mineral very hard and durable, combined with atmospheric particulate and smog, which rendered unreadable the *fresco*. Thanks to the willingness and open-mindedness of the restorers, a trial for the removal of superficial deposits, was performed using the biosurfactants as main cleaning agent.

Because traditional, mechanical and chemical, techniques for cleaning are ineffective or overly aggressive, it was decided to experiment an alternative way, based on the use of products of biological origin, for the purpose to recover the radiance of the original fresco.

Fig. 1.10: The Casina Farnese Loggia and a detail of the loggia's fresco (http://www.wmf.org/project/casina-farnese-palatine-hill).

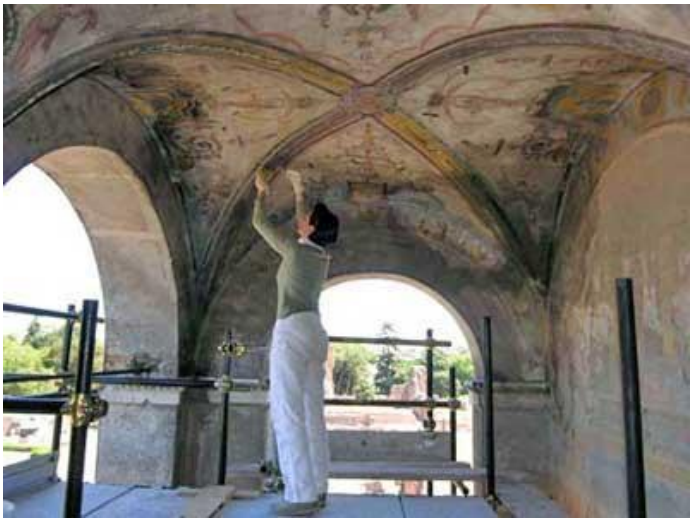


Fig. 1.11: A moment during the surface cleaning of the frescoes (http://www.wmf.org/project/casina-farnese-palatine-hill).

2. CHAPTER
AIM OF THE WORK

The possibility to develop new methods of restoration, which use products with low environmental impact, without toxic effects for operators and without negative consequences for artworks, selective for the treated material and cost-effective, can rely today on biotechnology. The multiple metabolic potential of microorganisms so far known is already a resource, which will however be amplified by the discovery of novel microorganisms and new molecules they produce.

This work aims to contribute to the search for new molecules of microbial origin for biorestoration, in order to offer an effective alternative or a suitable support to traditional methods, both chemical and mechanical, for those cases where these are not effective or require the use of potentially harmful products.

In particular, the research is focused on novel Surface Active Compounds (SACs) of microbial origin. These non-toxic and environmentally safe biopolymers exhibit a surface activity, and

have physico-chemical properties suitable for removing undesirable surface deposits from artistic surface.

Main objectives are:

- Screening of bacterial strains able to produce Surface Active Compounds and optimization of SACs production.
- Development of a purification procedure of produced SAC.
- Study of the emulsifying properties and antimicrobial characteristics of the SAC.
- Testing the cleaning capacity of the new product on artificial and real specimens.

The ultimate goal of the research is to provide a new product for the field of bio-restoration.

3. CHAPTER
MATERIALS AND METHODS

3.1 Bacterial strains

Bacterial strains were previously isolated from different sampling sites: from polluted sites (Sprocati et al. 2006), industrial wastewaters (Tasso et al. 2008) and archaeological sites (Sprocati et al. 2008) and belong to the ENEA-Lilith Strain Collection of the Microbiology Laboratory of Environmental Characterization, Prevention, and Recovery Unit of ENEA-Casaccia (Rome). The strains were identified (rDNA 16S sequencing) and characterized for biotechnological potential, in previous works (see tab. 3.1). The rDNA 16S sequences are deposited in GENBANK (http://www.ncbi.nlm.nhi.gov/BLAST).

Eleven strains were screened for their capacity in producing SACs (tab. 3.1). The strains were selected on the basis of two

essential features: non pathogenic and non spore forming bacteria, for the safety of the artwork and the operators.

Strain	Phylogenetic affiliation (% similarity of rDNA 16S)	GenBank Accession Number[a]	Source	References
TSNRS 4	*Ochrobactrum sp.* (100)	EU249585	Mercareccia Tomb of Tarquinia	[Sprocati et al. 2008]
MCC-A5	*Aeromicrobium erythreum* (98)	JF279932	Soil from an abandoned field located in Piana di Monte Verna (Naples, Italy)	[Sprocati et al. 2009]
MCC-SL5	*Duganella nigrescens* (99)	JF279923	Soil from an abandoned field located in Piana di Monte Verna (Naples, Italy)	[Sprocati et al. 2009]
MCC-Z	*Pedobacter sp.* (99)	JF279930	Soil from an abandoned field located in Piana di Monte Verna (Naples, Italy)	[Sprocati et al. 2009]
MCC-X	*Gordonia sp.*(99)	JF279928	Soil from an abandoned field located in Piana di Monte Verna (Naples, Italy)	[Sprocati et al. 2009]
MCC-S	*Massilia sp.* (99)	JF279920	Soil from an abandoned field located in Piana di Monte Verna (Naples, Italy)	[Sprocati et al. 2009]

MCC-E	*Micromonospora sp*.(99)	JF279912	Soil from an abandoned field located in Piana di Monte Verna (Naples, Italy)	[Sprocati et al. 2009]
MCC-G	*Nocardia sp*.(99)	JF279914	Soil from an abandoned field located in Piana di Monte Verna (Naples, Italy)	[Sprocati et al. 2009]
MCC-T	*Porphyrobacter donghaensis* (99)	JF279925	Soil from an abandoned field located in Piana di Monte Verna (Naples, Italy)	[Sprocati et al. 2009]
AGL17	*Acinetobacter calcoaceticus* (100)	EU118781	Abandoned contaminated site of Italsider- Bagnoli	[Sprocati et al. 2005]
CONC18	*Achromobacter xylosoxidans* (99)	EU275351	Sludge from the sedimentation of a sewage treatment plant of the tannery Ariston (NA)	[Tasso et al. 2008]
DSM-3257	*Bacillus subtilis* (100)			
DSM-1128	*Pseudomonas aeruginosa* (100)			

Table 3.1 List of the tested bacterial strains and their main features
a: the GenBank accession number of the tested strains.
The strains were identified by 16S rDNA sequence similarity with GENBANK data bank (http://www.ncbi.nlm.nhi.gov/BLAST)

Two strains of the DSMZ collection (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH) have been used as positive controls for biosurfactant/bioemulsifier production: *Bacillus subtilis* (DSM-3257) and *Pseudomonas aeruginosa* (DSM-1128).

3.2 Growth media

TSA (Tryptic Soy Agar) solid medium:

Bacto Tryptone (Pancreatic Digest of Casein) - 17 g/l

Bacto Sortone (Papaic Digest of Soyben Meal) - 3 g/l

Bacto Dextrose - 2,5 g/l

NaCl - 5 g/l

K_3PO_4 - 2,5 g/l

Glucose - 5 g/l

Agar - 15 g/l

Distilled H_2O 1000 ml

pH 7,3±0.2 a 25 °C

The medium was sterilized for 15 minutes at 121°C.

TSB (Tryptic Soy Broth) liquid medium:

Bacto Tryptone (Pancreatic Digest of Casein) - 17 g/l

Bacto Sortone (Papaic Digest of Soyben Meal) - 3 g/l

Bacto Dextrose - 2,5 g/l

NaCl - 5 g/l

K_2HPO_4 - 2,5 g/l

Distilled H_2O 1000 ml

pH 7,3±0.2 a 25 °C

The medium was sterilized for 20 minutes at 121°C.

Zhang-Miller (1992) medium:

NH_4Cl - 1,07 g/l

KCl - 1,50 g/l

Tris-HCl - 18,90 g/l

Glucose – 5 g/l

Peptone - 10 g

$MgSO_4 \cdot 7H_2O$ - 0,39 g/l

Distilled H_2O 1000 ml

pH 7,2 ± 0.2 a 25 °C.

The medium was sterilized for 20 minutes at 121°C and then were added 5 g/l of glucose.

Cooper (1981) medium:

NH_4NO_3 - 4,002 g/l

KH_2PO_4 - 4,08 g/l

Na_2HPO_4 - 7,12 g/l

$MgSO_4 \cdot 7H_2O$ - 0,197 g/l

$CaCl_2 \cdot 2H_2O$ - 0,0010 g/l

$FeSO_4 \cdot 7H_2O$ - 0,0011 g/L

Na_2EDTA - 0,0015 g/l

$MnSO_4 \cdot H_2O$ - 0,00017 g/l

Glucose – 40 g/l

Distilled H_2O 1000 ml

The medium was sterilized for 20 minutes at 121°C and then were added 40 g/l of glucose.

MSM (Mineral salt medium), described by Bodour *et al*. (2004):

Solution A contained (per liter)

$NaNO_3$ - 2.5 g

$MgSO_4 \cdot 7H2O$ - 0,4 g

NaCl - 1 g

KCl - 1 g

$CaCl \cdot 2H_2O$ - 0,05 g

H_3PO_4 (85%) - 10 ml

This solution was adjusted to pH 7.2 with KOH pellets.

Solution B contained (per liter)

$FeSO_4 \cdot 7H_2O$ – 0,5 g

$ZnSO_4 \cdot 7H_2O$ – 1,5 g

$MnSO_4 \cdot H_2O$ – 1,5 g

H_3BO_3 – 0,3 g

$CoCl_2 \cdot 6H_2O$ – 1,5 g

$CuSO_4 \cdot 5H_2O$ – 1,5 g

$NaMo_2O_4 \cdot 2H_2O$ – 0,1 g.

One milliliter of solution B was added to 1000 ml of solution A with 20 g/l of glucose, as the sole carbon and energy source, to form the MSM.

3.3 Screening for SACs production

The selected bacterial strains (Table 3.1) were inoculated (1:100) in 50 ml of two different media (Zhang-Miller and Cooper, described in a par. 3.2), from overnight TSB pre-cultures. Cultures were incubated aerobically at 37°C in rotatory shaker at 250 rpm and at 30°C at 200 rpm, respectively. The presence of SACs in the culture media was evaluated after 96 h of incubation by the Oil Spreading Test (Techaoei *et al*. 2007) and the Emulsification Assay (Cooper & Goldenberg 1987) in whole culture broths and cell-free supernatants, respectively, by centrifuging (9000*g*, 15 minutes) the culture broths and thereafter filtering with Millipore membranes (4.5 µm).

3.3.1 Oil Spreading Test

Biosurfactant activity of the tested strains was compared by measuring the diameter of the clear zones occurred when a drop of bioproduct-containing solution is placed on an oil-water surface. 50 ml of distilled water were added to a large Petri dish (15 cm diameter) followed by the addition of 20 µl of crude oil to the surface of water and 10 µl of supernatant or whole culture broth. The following step was measuring the diameter of clear zones coming from the triplicate assays from the same sample (Techaoei *et al*. 2007).

3.3.2 Emulsification Assay

The emulsifying capacity was evaluated by measuring the Emulsification Index E_{24}. The E_{24} of culture samples was determined by adding 2 ml of hexane and 2 ml of the whole culture broth/ cell-free broth in glass test tubes (15 mm), mixing with a vortex at high speed for 2 minutes and allowing to rest for 24h. The Emulsification Index, in percentage, was calculated by the following formula (Cooper & Goldenberg, 1987):

$$E_{24}\ \% = \frac{\text{Height of emulsion formed (mm)} \times 100}{\text{Total height of solution (mm)}}$$

The test was carried out on three replicates both for whole culture (W) and for cell-free supernatant (S).

3.3.3 Drop-collapse Assay

This is a method for a rapid and cheap qualitative evaluation of surface properties of SACs (Jain et al. 1991). One µl of methylene blue [0.1% (w/v)] was added to 20 µl of cell-free supernatant (S). The resulting mixture was spotted onto a piece of Parafilm sheet and photographed after 5 min. The methylene blue was added solely for visualization purposes and does not influence droplet collapse activity. Sterile culture medium was used as negative control and 1% SDS as positive control. The test was carried out on three replicates.

3.4 SACs production and partial extraction from batch cultures of the selected strain

Based on the results obtained from the screening, the growth and SACs production kinetics of the selected microbial strain were carried out according to the following procedure:

- 250 µl of the glycerol stock of the strain (stored at -80°C) was pre-inoculated in 10 ml of mineral salt medium (MSM;

Bodour et al. 2004) amended with 1% (w/v) glucose, as the sole carbon and energy source, in a 50-ml falcon and was incubated at 30°C in rotatory shaker at 130 rpm for 72h;

- the pre-culture was then inoculated 1:100 in 500 ml of MSM with different glucose percentages (0,25-0,5-1-2%, w/v), in 800 ml flasks;
- the culture broths were incubated under the same conditions for 96h;
- bacterial growth was evaluated by the plate count method, planting on TSA medium, and emulsification capacity by the Emulsification Index (E_{24}), both every 24h.

After 96 h of incubation, SACs produced were extracted from the culture broths as follows:

- the culture broth was centrifuged (9000 g, 30 minutes) to remove the cells and thereafter filtered with Millipore membranes (4.5 µm);
- the filtered supernatant was added with half volume of hexane, shaken and allowed to stand for 4 h at room temperature;
- the produced foam was collected after four water washes, evaporated to dryness to remove residual hexane and then lyophilized.

The lyophilized crude extract was re-dissolved in sterile water at different concentrations.

To correlate the concentration of the crude extract to the emulsification index (E_{24}), serial dilutions of the extract were tested (0-0,05-0,075-0,15-0,3-0,6-1,2-2,5-5-10 mg/ml in water).

3.5 SACs characterization

3.5.1 Chemical characterization

For a preliminary characterization, colorimetric assays were carried out to determine protein and carbohydrate content.

Protein assay

In order to measure the protein content of the lyophilized crude extract the dye-binding assay of Bradford (1976) was used. It is based on the observation that Coomassie Brilliant Blue G-250 exists in two different colour form, red and blue. The red form is converted to the blue form upon binding of the dye to protein. The protein-dye complex has a high extinction coefficient thus leading to great sensitivity in measurement of the protein.

Standard curve was built with different concentrations of BSA (bovine serum albumin) : 0, 0.05, 0.1, 0.15 and 0.2 µg/ µl. Nine hundred µl of Bradford solution were added. The absorbance at 595 nm was measured after 30 minutes. BSA quantity was plotted against the corresponding absorbance resulting in a standard curve used to determined the protein content in the samples.

Different amounts of the lyophilized crude extract were tested: 15, 25, 50 and 100 µg.

Using the standard curve, protein content of the samples was extrapolated.

Carbohydrate assay

The presence of carbohydrate groups in the bio-molecule was assayed with the method described by Dubois et al. (1956), a simple, rapid and sensitive colorimetric determination of sugars. It is based on the capacity of the simple sugars, oligo and polysaccharides, including the methyl ethers with free or potentially free reducing groups, to give an orange-yellow colour when treated with phenol and concentrated sulphuric acid.

Standard curve was built with different concentrations of the standard sugar: 10, 50 and 100 µg of glucose in distilled water to 175 µl. The same amount (175 µl) of phenol and 900 µl of concentrated sulphuric acid (95%) were added to each concentration.

The colour developed at a constant phenol concentration is proportional to the amount of sugar present. The absorbance was measured at 490 nm. Glucose content was plotted against the corresponding absorbance resulting in a standard curve used to determined the sugar content in unknown samples.

Different amounts of the lyophilized crude extract were tested. Using the standard curve, carbohydrate content of the sample was extrapolated.

SDS-PAGE

Proteins were analyzed by 12% SDS-PAGE. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (**SDS-PAGE**) is a technique used for separating proteins based on their ability to move within an electrical current, which is a function of the length of their polypeptide chains or of their molecular weight. The gel was prepared as follows:

Separating Gel (5 ml)

distilled water – 2,15 ml

1,5M Tris-HCl, pH 8.8 – 1,25 ml

10% SDS – 50 µl

40% Stock Acryl-bis – 1,5 ml

10% APS – 50 µl

TEMED – 5 µl

Stacking Gel (5 ml)

distilled water – 3,5 ml

0,5M Tris-HCl, pH 6.8 – 1,25 ml

10% SDS – 50 µl

40% Stock Acryl-bis – 500 µl

10% APS – 50 µl

TEMED – 5 µl

The electrophoresis gel was run in a BioRad apparatus (Mini Protean) at 90 mV for 2 hours. BSA was used as standard.

At the end of the run the gel was fixed in a Methanol:Acetic Acid:Water solution (30:10:60) for 30 minutes and then stained with 0.25% Coomassie Brilliant Blue R-250 until the gel is a uniform blue color. *This method will detect as little as 0.1µg/band.* The gel was destained for 4 – 24 hours in the same fixing solution. Bands will begin to appear in 1 – 2 hours. In alternative, Silver Staining was performed according to Pharmacia kit instructions.

3.5.2 Study of SACs stability

The stability of the lyophilized crude extract to temperature, pH and electrolyte effects was studied with the following tests

(Burgos-Dìaz et al. 2011). A stock solution at a concentration of 1mg/ml was prepared, dissolving the lyophilized crude extract in distilled water. The Emulsification Assay (E_{24}) was carried out with the procedure described above (par. 3.3.2)in order to test the effect of different parameters on the emulsification capacity of the extract, , after subjecting the extract to the following treatments:

Temperature effect: to determinate the thermal stability, 2 ml from the stock solution were maintained at different temperatures (-80, +7, +25, +37, +70, +121°C) for 30 minutes. Then the E_{24} test was carried out.

Effect of pH change: to determinate the effect of pH on SACs activity, 2 ml from the stock solution were adjusted at pH values (2, 3, 4, 7, 9, 10, 11 and 12), with HCl and NaOH. After 30 minutes, the E_{24} test was carried out for each pH condition.

Electrolyte concentration effect: The effect of addition of different concentration of NaCl on the emulsion production was investigated. The extract was re-dissolved in distilled water containing increasing concentrations of NaCl (5-10-15-20%, w/v).

Another analyzed parameter was the stability of the emulsion produced by crude extract in the course of time. A sufficient quantity of emulsion was produced by mixing 2 ml of crude extract (1 mg/ml in water) with 2 ml of hexane and it was hand shaken for several minutes. The emulsion was picked up and transferred in a glass tubes

(15 mm); the height of the emulsion was measured every 7 days for 4 months.

All the tests were carried out on three replicates.

3.5.3 Surface tension measurement

The lyophilized crude extract was solubilised in Ultra-pure MilliQ water at the concentration of 7 mg/ml. The surface tension was determined by the Du Noüy ring method using a 3S tensiometer (GBX, Romans sur Isère, France). The MSM medium was used as negative control. All determinations were performed in triplicate.

3.5.4 Emulsifying properties

The ability of the SACs to emulsify olive oil and some hydrocarbons such as hexadecane, cyclohexane, isooctane, diesel, xilene, toluene, and crude oil was determined. The Emulsification Assay (E_{24}) was carried out on a set of three replicates, using 2 ml of each hydrocarbon in place of hexane. As reference, we used chemical surfactants (at the same concentration): Tween 20, Tween 80 and Triton X-100 (Tuleva et al. 2007).

3.6 Anti-microbial activity of the SACs

Eight bacterial strains, belonging to ENEA-*Lilith* strain collection, were selected to test the anti-microbial activity of the SACs (see table 3.2).

Strain	Identification, similary (%) rDNA 16S and sequence number	GenBank Accession Number[a]	Source	References
TSNRS12	*Rhodococcus erythropolis (100)*	EU249592	Mercareccia Tomb of Tarquinia	[Sprocati et al. 2007]
DPBS17	*Bacillus megaterium (100)*	EU249559	Mercareccia Tomb of Tarquinia	[Sprocati et al. 2007
DAN10	*Bacillus pumilus (100)*	EU249560	Mercareccia Tomb of Tarquinia	[Sprocati et al. 2007]
TPBF14	*Bacillus cereus (100)*	EU249556	Mercareccia Tomb of Tarquinia	[Sprocati et al. 2007]
TPSB9	*Paenibacillus sp. (97)*	EU249589	Mercareccia Tomb of Tarquinia	[Sprocati et al. 2007]
TPBF11	*Cellulomonas sp. (99)*	EU249577	Mercareccia Tomb of Tarquinia	[Sprocati et al. 2007]

LAM21	*Acinetobacter calcoaceticus (100)*	EU118781	Abandoned contaminated site of Italsider- Bagnoli	[Sprocati et al. 2005]
CONT-Z	*Rhodococcus sp. (99)*		Isolated during air monitoring of ENEA laboratory	

Table 3.2 List of bacterial strains tested for SAC's anti-microbial activity
a: the accession number to GENBANK for each tested strain.
The strains were identified by 16S rDNA sequence similarity with GENBANK data bank (http://www.ncbi.nlm.nhi.gov/BLAST).

The screening was performed using 96 wells microplates and evaluating the kinetics of growth of the different microbial strains in presence of various extract concentrations, through the measurement of the absorbance (600 nm) in each well.

The experimental procedure is described as follows:

- the extract was suspended in TSB medium to obtain three stocks at different concentrations: 0,05 – 0,5 mg/ml - 1 mg/ml, that were aliquoted in the wells (250 µl);

- 25 µl of each bacterial suspension in TSB, corresponding to 10^7 CFU/ml, were inoculated in each well;

- the microplates were incubated at constant R.H. conditions, at 28°C. The spectrophotometric reading of the plates was performed after 0, 24, 48, 120 and 144 hours of incubation.

Each strain was tested in duplicate with the three SAC's concentrations. Absorbance values were compared to that obtained in TSB without added SAC's.

3.7 Cleaning trials on lab-made samples

Preparation of samples

In order to carry out *in vivo* bio-cleaning trials, lab-made samples were prepared. The samples were made up of bricks of 11x24cm, covered with a plaster layer.

The brick surface was divided in five areas of 5x11 cm. Olive oil and animal fat (lard) were spread by swab each on two areas of the surface of the brick (fig. 3.2-3.3). The fifth area was not greased and represents the blank.

Another brick was exposed to the fumes of burning oil, fat and kerosene in succession to produce the blackening and fat deposits on their plaster surface (fig. 3.4).

In order to accelerate the aging, the samples were kept in a climatic chamber set at 70°C for several months (up to six months).

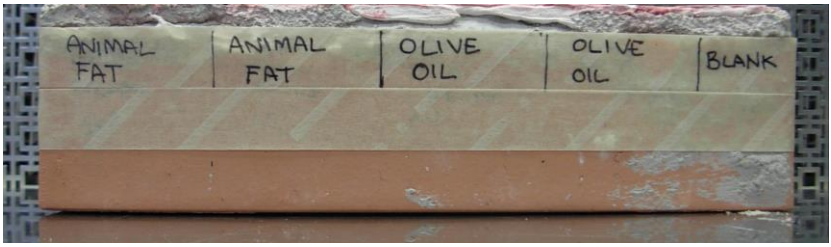


Fig.3.2: A lab-made brick covered with animal fat (areas 1 and 2) and olive oil (areas 3 and 4), before the treatment.

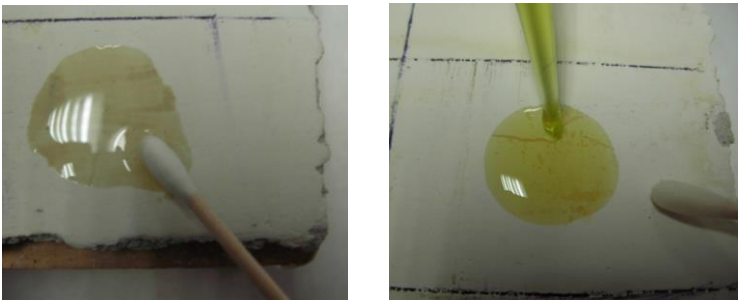


Fig.3.3: application of animal fat (left) and olive oil (right), on the plaster surface of the bricks.

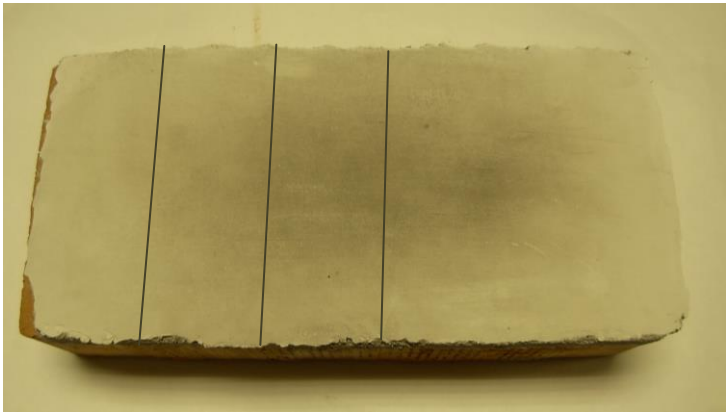


Fig.3.4: A lab-made sample with fume deposits of olive oil, lard and kerosene before the treatment.

Cleaning trials

A stock solution containing 2 mg/ml of the lyophilized crude extract was prepared in water. As optimal carrier for the application

of SACs was selected a colloidal clay (Laponite RD) that in water swells to produce a thixotropic, heat stable, clear gel. The gel was prepared at 8% of laponite, added with 6% of SAC stock solution. The gel was spread with a spatula on the surface of different bricks (fig. 3.5) and covered with plastic wrap. After 2, 4 and 6 hours, the gel was removed with a spatula and the residues were washed out with a wet brush (fig. 3.6).

A gel of laponite 8% in water was applied in a nearby area as negative control (fig. 3.5).

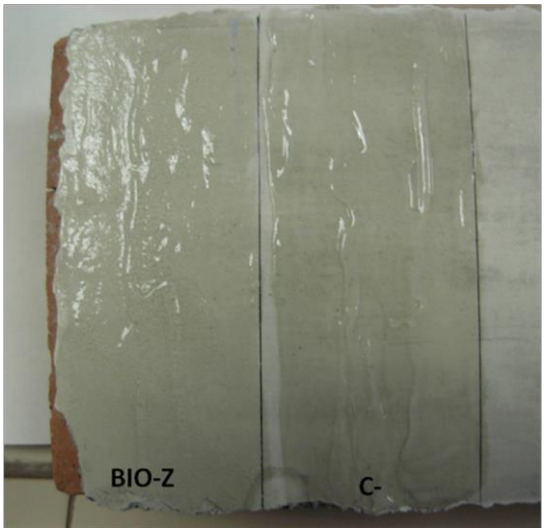


Fig. 3.5: Gel application on the surface of artificial aged bricks with fume deposits. BIO-Z = laponite containing Bio-Z; C-= laponite in water as negative control.

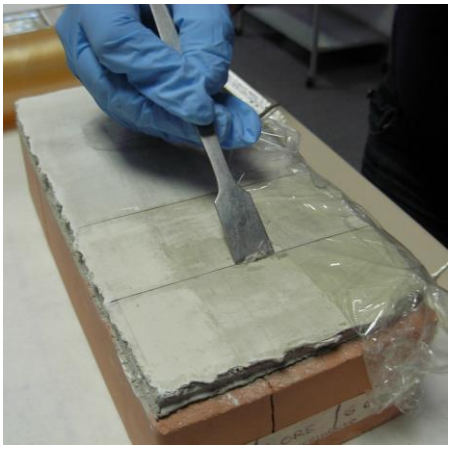


Fig.3.6: Removal of the gel after the application on the brick sample with fume deposits.

3.8 Evaluation of the treatment efficacy

In order to evaluate the cleaning efficacy, the color changes after treatment were analyzed by means of visual inspection under visible and UV light, by colorimetric measurements and staining with selective dyes.

The UV observations were performed with Fluorescent Lamp Electronic of Wood (G23 fluorescent tube, 9W, compact UV light beam 360-380 nm).

Colorimetric measurements were carried out with a spectrophotometer Techkon SP 820λ (10°/ D 65 light), measuring the *L*a*b** values according to the CIELAB 1976 system. Fiftheen points of each sample were analysed before and after the treatment.

Chromatic coordinates were measured for each condition and each measure was the mean of 3 determinations. The colorimetric system CIE 1976 provides the following interpretation of change:

- an increase of L* coordinate means a higher brightness of the sample in the examined area;
- a decrease of the a* parameter moves the colour in the green direction;
- a decrease of the b* component moves the colour in the blue direction;

The parameter ΔE* represents the overall colour variation and is calculated by the following equation:

$$\Delta E^* = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$$

where $\Delta E^* > 5$ is perceived by the human eye (Palazzi 1995).

Olive oil and animal fat removal was evaluated staining the brick surface with Sudan III, a dye which selectively binds lipids. The dye (0.1% w/v in ethanol) was spread on the brick surface and then washed out with ethanol (96%).

3.9 Cleaning trials on fresco paintings "*The story of Hercules and Cacus*" at Casina Farnese

During the restoration of the fresco paintings at Casina Farnese, a trial for the removal of superficial deposits, a thick and complex layer of smog, atmospheric particulate and casein, was performed employing the extracted SACs as main cleaning agent.

As described for the experiments conducted in laboratory, a stock solution containing 2 mg/ml of the crude extract was prepared in water. Laponite RD was selected again as carrier for the application of SACs. The gel was prepared at 8% of laponite, added with 6% of SAC stock solution. The gel was spread with a spatula on the *fresco* surface, previously washed with a wet sponge, and covered with Saran wrap (fig. 3.7). After 18 hours, the gel was removed with a spatula and the residues were washed out with a wet brush.

A gel of laponite 8% in water was applied in a nearby area as negative control (fig. 3.7).

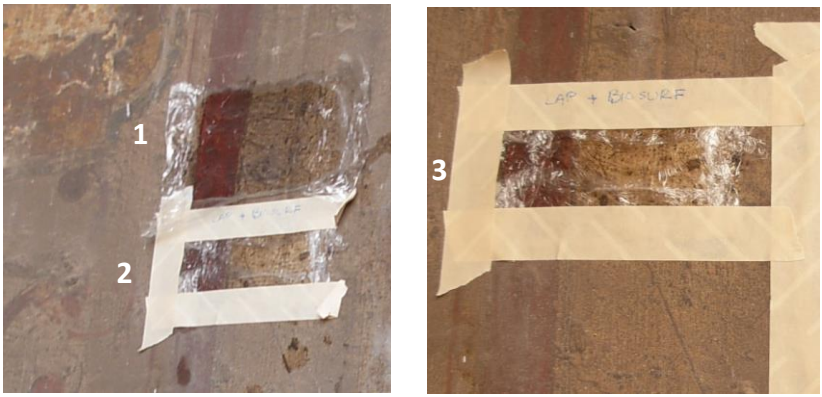


Fig. 3.7: Applications of SACs on the *fresco* surface in Casina Farnese lodge: negative control laponite without SACs (1), laponite with SACs stock solution (2-3).

4. CHAPTER
RESULTS

4.1 Screening of microbial strain for SACs production

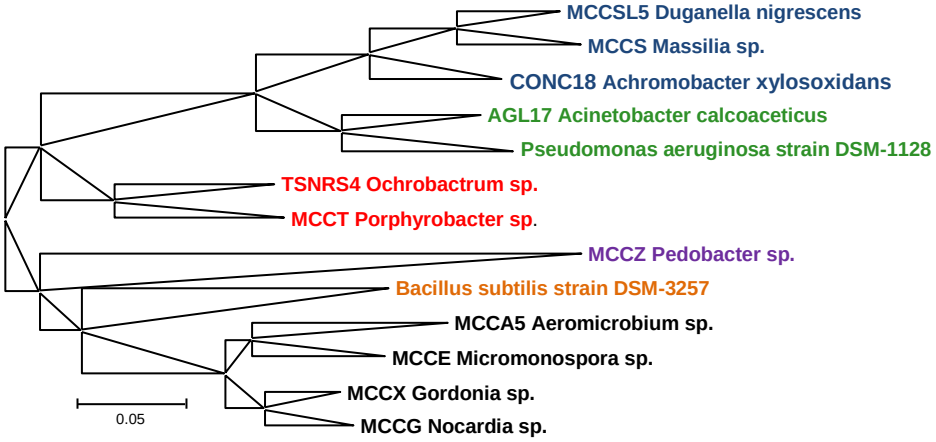
The aim of the screening was a focused on identifying bacterial strains able to produce biopolymers with strong superficial and interfacial properties (Surface Active Compounds, SACs).

The eleven bacterial strains selected belong to six different phylogenetic groups (Alphaproteobacteria, Actinobacteria, Bacilli, Betaproteobacteria, Flavobacteria, Gammaproteobacteria) as shown in the phylogenetic tree in fig.4.1.

The production of SACs was evaluated by the Oil Spreading Technique (OST; Techaoei *et al*. 2007) and the Emulsification Assay (EA; Cooper and Goldenberg 1987), described in detail in cap.3 par. 3.1, performed on whole culture broths and cell-free supernatants respectively, and by the Drop-Collapse Assay (DC; Jain et al. 1991) on cell-free supernatants. The tests were carried out every 24h on batch cultures prepared in two different culture media (Zhang-Miller medium and Cooper medium, described in cap.3.2).

Results obtained for the different strains are shown in table 4.1.

Pseudomonas aeruginosa and *Bacillus subtilis* were used as positive controls.



α-Proteobacteria β-Proteobacteria γ-Proteobacteria Actinobacteria Bacilli Flavobacteria

Fig. 4.1 – Phylogenetic tree of the bacterial strains used, obtained by the program MEGA 4.1 (Molecular Evolutionary Genetics Analysis) using Neighbor-joining algorithm (Saitou & In 1987).

Strain code	Species identification	Zhang-Miller culture medium					Cooper culture medium				
		E_{24}% [a]		OST[b]		DC[c]	E_{24}% [a]		OST[b]		DC[c]
		W	S	W	S	S	W	S	W	S	S
TSNR4	*Ochrobactrum sp.*	**NG**					**12**	**16**	+	+	**2**
MCCA5	*Aeromicrobium erythreum*	**12**	**12,5**	+	+	**2**	**22**	**17**	+	+	**2**
MCCSL 5	*Duganella nigrescens*	**NG**					**NG**				
MCCZ	*Pedobacter sp.*	**NE**	**NE**	+	+		**54**	**56**	+	+	**4**
MCCX	*Gordonia sp.*	**25**	**4**	+	+	**3**	**52**	**29**	+	+	**3**
MCCS	*Massilia sp.*	**NG**					**NG**				
MCCE	*Micromonospora sp.*	**NE**	**NE**				**9**	**NE**			**2**
MCCG	*Nocardia sp.*	**28**	**NE**	+	+	**2**	**4**	**NE**	+	+	**2**
MCCT	*Porphyrobacter donghaensis*	**NG**					**NG**				
AGL 17	*Acinetobacter calcoaceticus*	**48**	**12**	+++	++	**3**	**43**	**10**	+	+	**3**

CONC 18	*Achromobacter xylosoxidans*	**32**	**48**	+	+	**3**	**48**	**52**	+	+	**3**
DSM-3257	*Bacillus subtilis*						**36**	**4**	+++ +	+++ +	
DSM-1128	*Pseudomonas aeruginosa*	**8**	**44**	+++ ++	+++ ++						
C+						**5**					**5**
C-						**2**					**2**

Table 4.1: Results of the Emulsification Assay, Oil Spreading Test and Drop-collapse assay (diameter) obtained on whole culture broths (W) and cell-free supernatants (S) for the screened bacterial strains on the two culture media. Reported values represent the highest result for each strain at 96 h of growth.

a: emulsifying activity was expressed as E_{24}%= the percentage of the volume occupied by the emulsion compared to the total volume.

b: diameter of the oil drop expressed in mm: "+"=1- 5 mm; "++"= 6 -10 mm; "+++"= 11-20 mm; "++++"= 21-50 mm,;"+++++"= > 51 mm.

c: diameter of the collapsed drop, expressed in mm; positive (C+) and negative controls (C-) were culture medium with and without 1% SDS.

NG: no growth, NE: no emulsifying activity

Results showed that some strains were able to express an emulsifying activity near or above 50%, values that are higher than those obtained with the reference strains *Bacillus subtilis* and *Pseudomonas aeruginosa*, known as biosurfactant producers. None of the selected strains showed OST and DC higher than the reference strains (tab.4.1). Four bacterial strains were potentially interesting for this research: *Acinetobacter calcoaceticus* (AGL 17) and *Achromobacter xylosoxidans* (CONC 18) in both culture media; *Gordonia sp*. (MCCX) and *Pedobacter sp.* (MCCZ) in Cooper medium.

Out of the tested strains, MCC-Z *Pedobacter sp*. was selected for providing the best emulsification activity (E_{24} = 54-56%, see tab.4.1), in both whole culture (W) and cell-free supernatant (S). Moreover the strain MCC-Z produced the highest effect of drop-collapse (fig. 4.2).

Pedobacter sp. (MCC-Z) is a Gram negative Sphingobacteriaceae, a non-flagellated heterotrophic bacterium, non pathogen and non spore-forming, producing rod shaped, pink-colored colonies (fig.4.3).

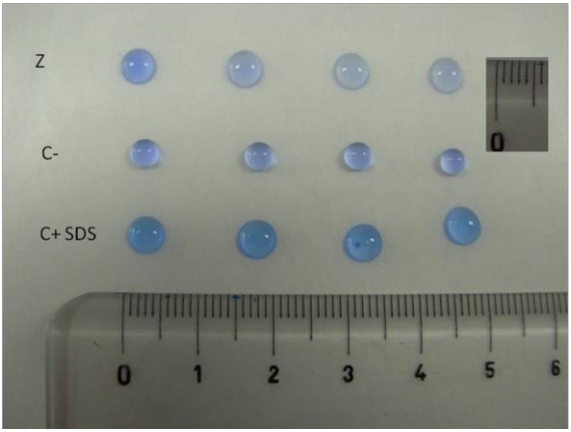


Fig. 4.2: Drop-collapse assay by the strain MCC-Z; cell-free supernatant (Z), negative and positive controls (C- and C+).

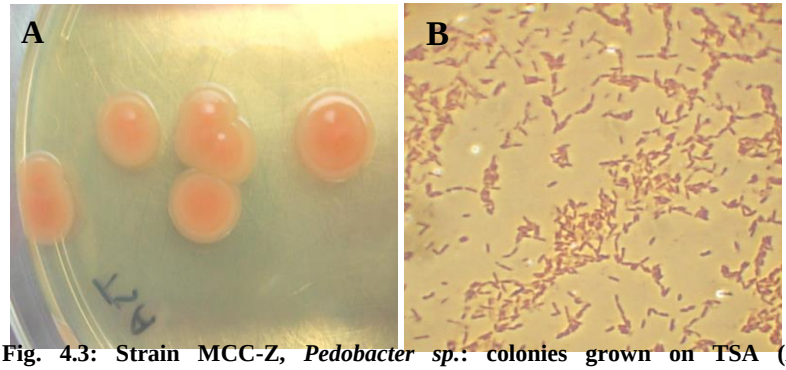


Fig. 4.3: Strain MCC-Z, *Pedobacter sp.*: colonies grown on TSA (A); microscope image of Gram staining (100x) (B).

4.2 MCC-Z growth curve and SACs production kinetics

The selected strain, MCC-Z *Pedobacter sp*., was grown on a mineral salt medium, MSM (the composition is reported cap. 3.2; Bodour *et al*. 2004), described in the literature as the best culture medium for SACs production in Flavobacteriaceae. Indeed the strain produced E_{24} values (E_{24} 59-64%) higher than those obtained in Cooper medium.

The optimization for SACs production was conducted in MSM using glucose at different percentages (0,25-0,5-1-2%, w/v) as only carbon source. The emulsification assay was performed every 24h, for each experimental condition. Graphics in fig.4.4 the optimal concentration of glucose is 0,5% (w/v), producing an E_{24} value (at 96h of growth) of 64% (fig.4.4-4.5).

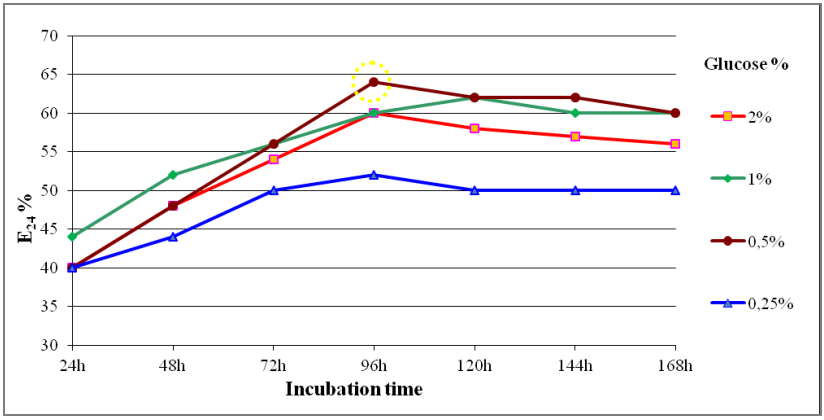


Fig. 4.4: E_{24}% values of cell-free supernatants of MCC-Z cultures in MSM with different glucose concentrations.

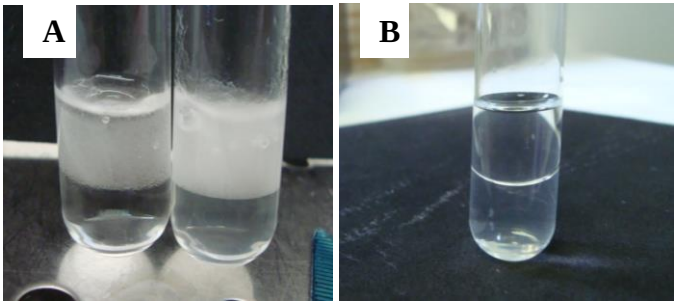


Fig. 4.5: Emulsification Assay on MCC-Z supernatant after 96 hours of growth in MSM added with 0,5% w/v glucose. Supernatant free of cells + hexane (A) E_{24} = 64%; MSM medium + hexane as blank (B).

In this condition the kinetics of SACs production has been studied in relation to the bacterial growth (fig. 4.6). Results showed that the strain MCC-Z excretes the SACs in the medium during the exponential phase (24h - 48h) and the bio-product concentration reaches the maximum during the stationary phase of the growth (96 hours).

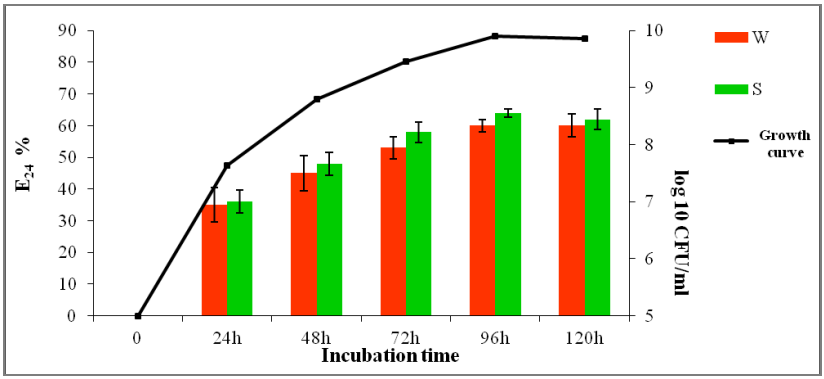


Fig. 4.6: Growth curve and emulsifying activity of MCC-Z; "W": whole culture broths and "S": cell-free supernatant. Samples were taken at 24h intervals and the values reported are the average of three replicates ± the standard error.

An important factor in consideration of a possible application of biosurfactant ,is its positioning in relation to the bacterial cell Tests E_{24} conducted under the different conditions (Fig. 4.7) allowed us to establish that the biosurfactant produced by MCC-Z is of t extracellular type, then it is produced and released outside the cell in growth medium.

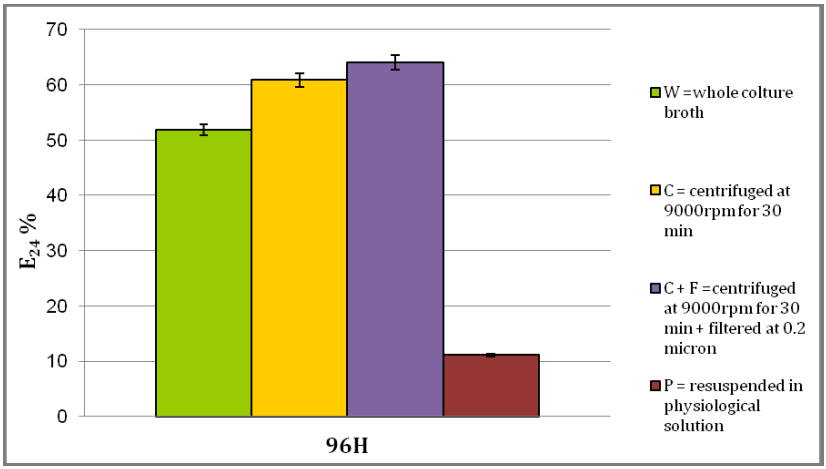


Fig. 4.7: E_{24} values under different condition at 96 hours of incubation ± the standard error.

The culture broth was then centrifuged and filtered as described in the previous chapter (3.4), subsequently a preliminary extraction with hexane and a process of lyophilization were performed. The lyophilized crude extract, called *Bio-Z*, retains its emulsifying power for long, as demonstrated by repeat testing E_{24} (E_{24} = 62-64%). The yield of the process is about 150 mg of *Bio-Z* per liter of culture.

4.3 SACs characterization

4.3.1 Chemical characterization

The colorimetric assays, described in cap. 3.5.1, using the method of Dubois (1956) for carbohydrates and the Bradford method (1976) for proteins, have been conducted on the lyophilized crude extract suspended in distilled water. Compositional analysis revealed that the crude extract *Bio-Z* consists for about 98% of carbohydrates and a minor fraction of protein (less than 1%). The considerable amount of glucose found in the crude extract was presumably due to the presence of glucose residues in the culture broth co-extracted with *Bio-Z*.

SDS-PAGE

Lyophilized crude extract was analyzed by SDS-PAGE for protein content. The electrophoresis gel was performed according to the procedure described in the previous chapter (3.5.1).

By comparing *Bio-Z* with the band produced by the BSA (Bovine Serum Albumin), used as reference for molecular weight (66,5 kDa), the major protein component of *Bio-Z* results approximately 60 kDa, as it can be observed in fig. 4.8.

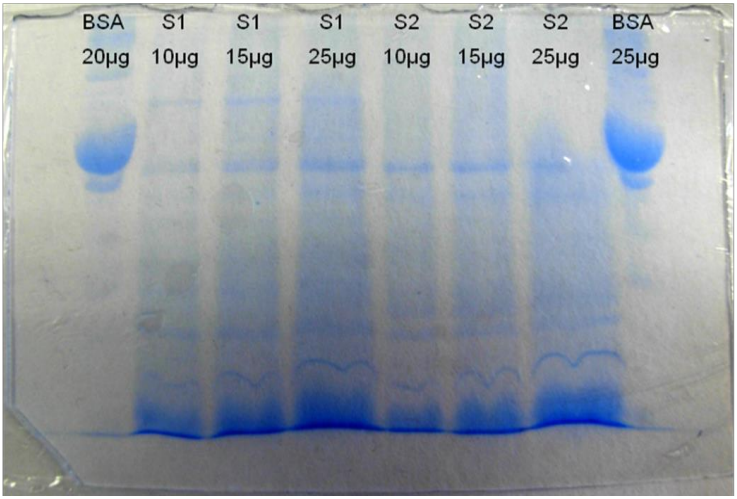


Fig.4.8: SDS-Page of *Bio-Z*; S1 and S2=*Bio-Z* samples, BSA=standard (PM: 66.5 Kdalton).

4.3.2 Stability of the SACs

In view of a real application of *Bio-Z* in the field of bio-restoration, it is essential to assess the quality of the crude extract, and especially its stability by changing certain parameters (temperature, pH and salinity). Stability was studied measuring changes in the Emulsification Index E_{24}%.

Temperature.

The stability of the crude extract at the concentration of 1 mg/ml was tested over a range of temperatures, from -80 °C to 120°C (fig. 4.9). *Bio-Z* is thermostable showing a slight flection with

increasing temperatures; only the autoclaving at 121°C caused a reduction of E_{24} value.

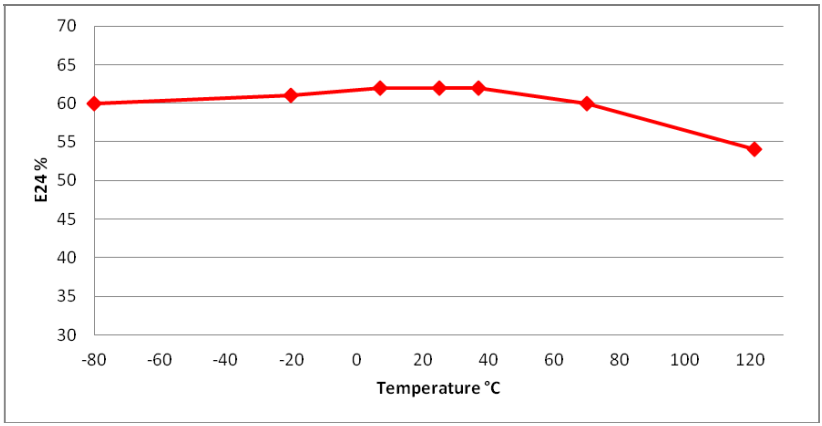


Fig.4.9: Effect of temperature on emulsification activity of *BioZ*.

pH.

Fig. 4.10 shows the effect of pH on *Bio-Z* emulsification activity. The range of pH values where Bio-Z stays stable is between 3 and 11.

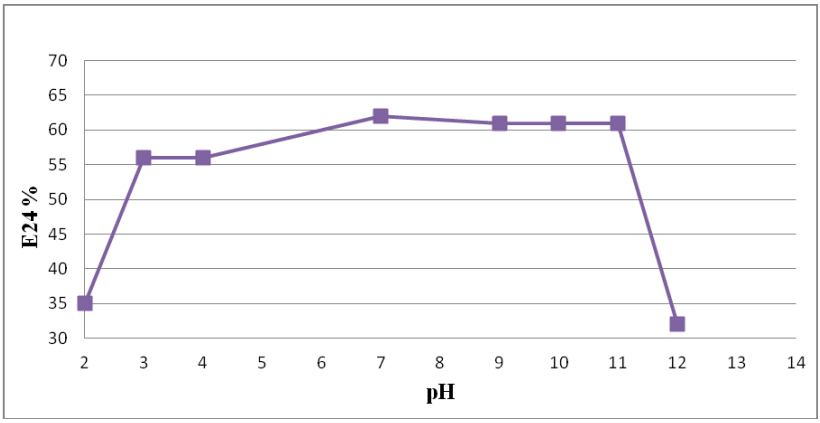


Fig.4.10: Effect of pH on emulsification activity.

Electrolyte concentration.

Fig. 4.11 shows that sodium chloride concentrations ranging between 5 and 20% w/v had no significant effect on the emulsification index .

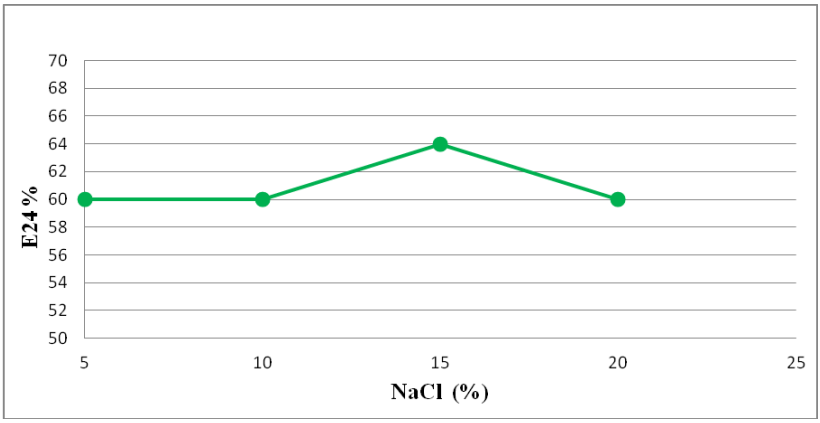


Fig.4.11: Effect of salinity on emulsification activity.

The stability of the produced emulsion over time was also demonstrated.

The monitoring of the emulsion volume (the procedure is described in cap. 3.4) with time is shown in figure 4.12. Results of the measures, done in three replicates, showed that the emulsion is stable and it remains almost constant over four months; and after 17 weeks a slight reduction (22 to 20 mm) was observed.

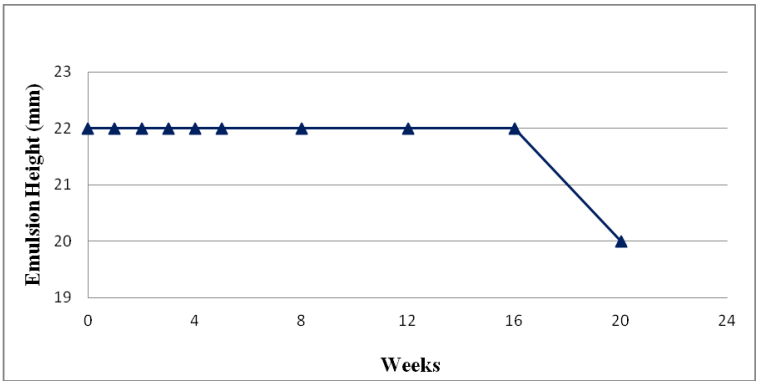


Fig.4.12: Emulsion stability in time: the emulsion volume (expressed as the height in mm occupied by the emulsion in a vial) was measured every week for 5 months.

4.3.3 Surface tension measurement

The measurements of the surface tension of the crude extract showed that *Bio-Z* has a good surfactant activity, lowering the surface tension of about 40 mN/m (fig. 4.13), starting from low concentrations (0,5 mg/ml), and then stabilized at concentrations between 2 and 3 mg / ml.

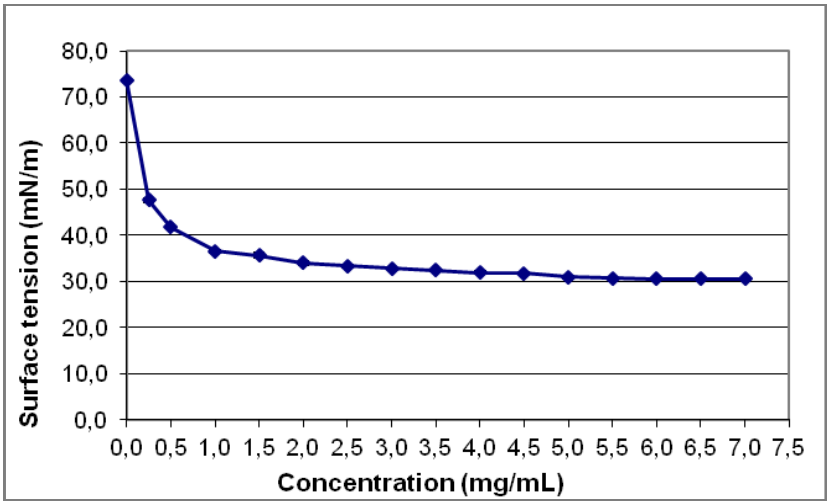


Fig.4.13: The surface tension in function of the different concentration of Bio-Z. The values reported are the mean of three measurements

4.3.4 Emulsifying properties

In the context of the SAC characterization, a series of experiments were prepared to evaluate the emulsifying properties. In particular, to verify the proportionality between emulsifying activity and concentration of the bio-product, has been constructed a curve of values E_{24} (%) in function of the concentrations of the lyophilized crude extract, re-suspended in distilled water. Observing the figure 4.14, it can be seen for lower concentrations of bio-Z (up to about 1 mg/ml), there is almost a linear proportionality (E_{24} values from 0 to

58%); in the range between 1 and 10 mg/ml, the E_{24} values do not show, other hand, a corresponding increase (from 60% to 70%).

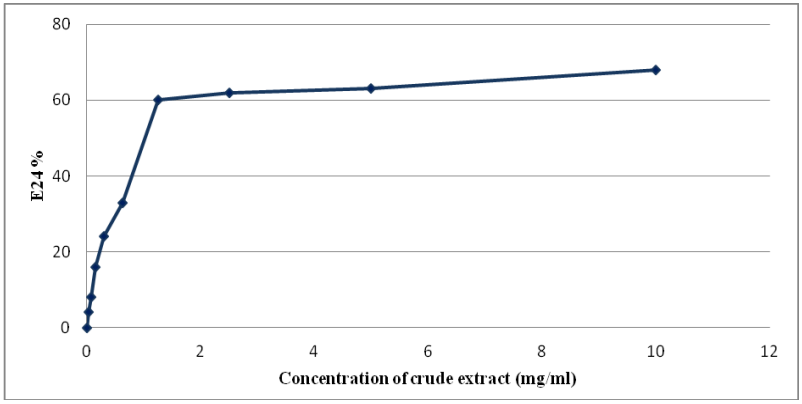


Fig.4.14: The concentration of lyophilized crude extract as a function of E_{24}%.

Another interesting parameter to characterize our SAC (Tuleva *et al.* 2007) is the ability to emulsify different hydrocarbons. As shown in table 4.2, the emulsifying capacity of *BioZ* was compared with the one of the chemical surfactants Triton X-100, Tween 20 and Tween 80 against olive oil and different hydrocarbons. The crude extract efficiently emulsified all hydrocarbons (E_{24}=64% - 68%), except for olive oil and diesel, with an activity higher than the chemical surfactants. Only the crude oil does not allow an accurate reading of the height of the emulsion.

HYDROCARBONS	E_{24} %			
	Bio-Z	Triton X-100	Tween 20	Tween 80
olive oil	44	56	60	60
diesel	44	63	61	59
xilene	64	61	62	59
toluene	68	35	28	32
hexadecane	64	60	57	59
isooctane	68	54	58	54
cyclohexane	64	48	48	44

Table 4.2: Emulsification assay (E24%) carried out in a set of three replicates, using different hydrocarbons in place of hexane.

4.4 Anti-bacterial activity

The anti-bacterial activity of *Bio-Z* was evaluated on eight bacterial strains, according to the procedure described in cap. 3.6, in terms of bacterial growth inhibition.

Growth curves for the strains TPBF 11 (*Cellulomonas sp*.) and TPBF 14 (*Bacillus cereus*) showed that the presence of *Bio-Z*, in the culture medium, even at low concentrations, has a strong inhibitory effect on bacterial growth (fig. 4.15 - 4.16).

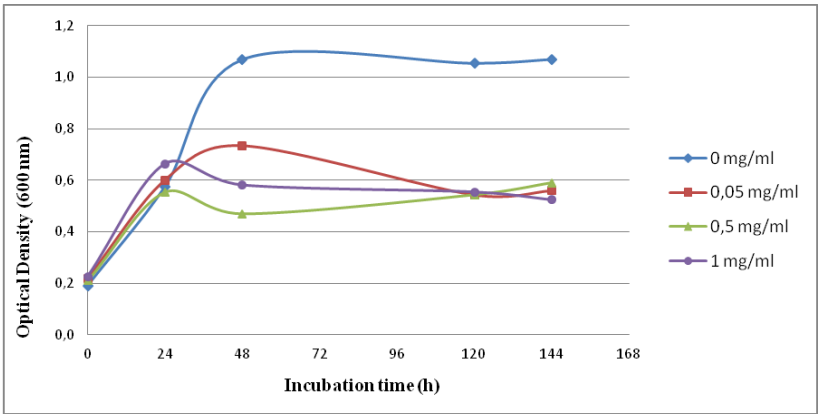


Fig.4.15:Growth curve of TPBF 11(*Cellulomonas sp*.) in presence of various bio-Z concentrations (0,05-0,5-1 mg/ml).

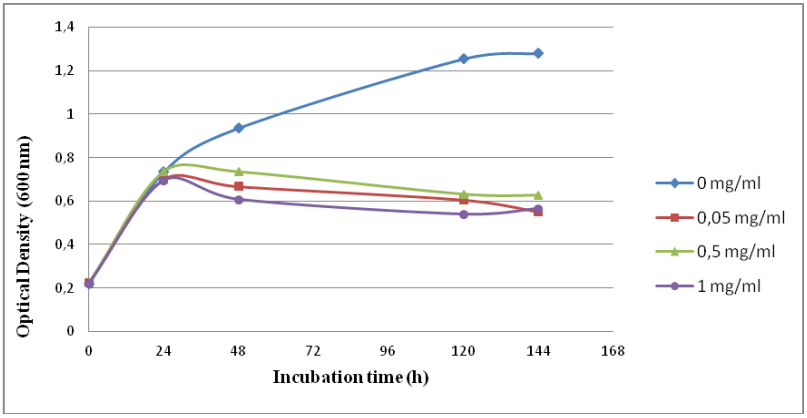


Fig.4.16: Growth curve of TPBF 14 (*Bacillus cereus*) in presence of various bio-Z concentration (0,05-0,5-1 mg/ml).

Regarding the strains DAN 10 (*Bacillus pumilus*) and DPBS 17 (*Bacillus megaterium*), the presence of bio-Z inhibits their growth only at the highest concentration (1 mg/ml; fig. 4.17 - 4.18).

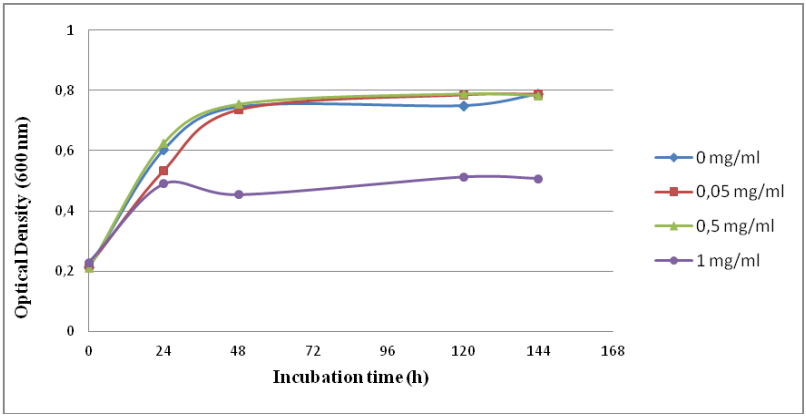


Fig.4.17: Growth curve of DAN 10 (*Bacillus pumilus*) in presence of various bio-Z concentration (0,05-0,5-1 mg/ml).

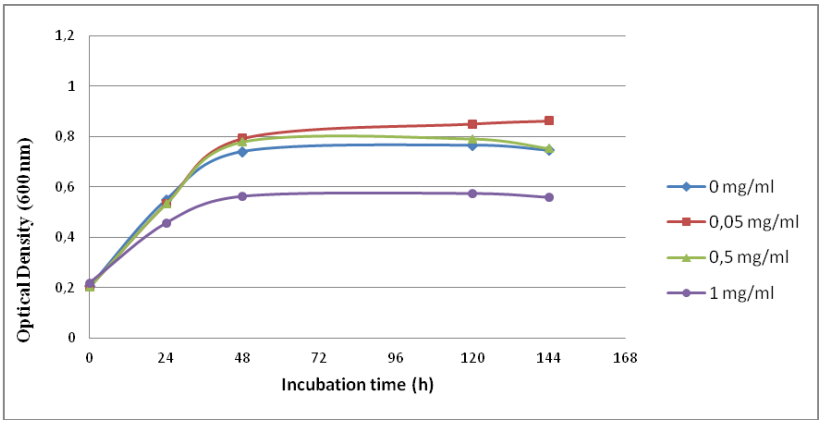


Fig.4.18: Growth curve of DPBS 17 (*Bacillus megaterium*) in presence of various bio-Z concentration (0,05-0,5-1 mg/ml).

On the contrary the presence of *Bio-Z* had either no influence or a slightly positive effect on the growth of LAM 21 (*Acinetobacter calcoaceticus*), TSNRS 12 (*Rhodococcus erytropolis*) and CONT-Z (*Rhodococcus sp.*) (fig. 4.19 - 4.20 – 4.21).

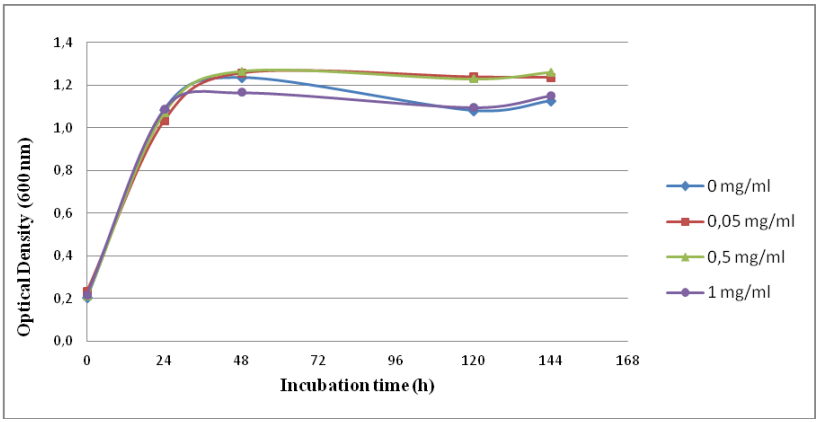


Fig.4.19: Growth curve of LAM 21 (*Acinetobacter calcoaceticus*) in presence of various bio-Z concentration (0,05-0,5-1 mg/ml).

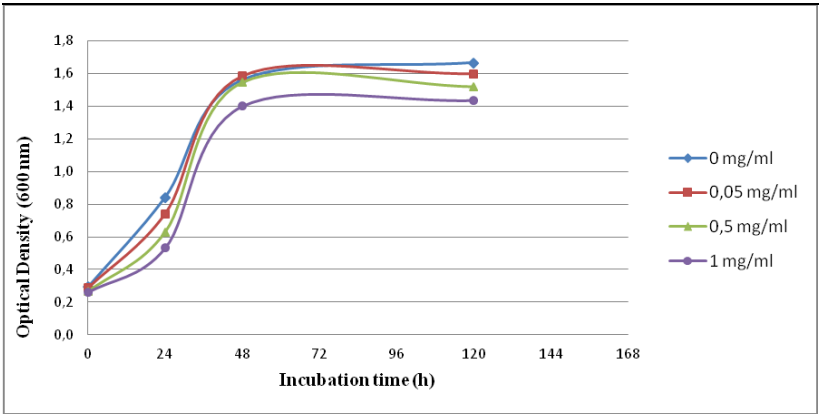


Fig.4.20: Growth curve of TSNRS 12 (*Rhodococcus erytropolis*) in presence of various bio-Z concentration (0,05-0,5-1 mg/ml).

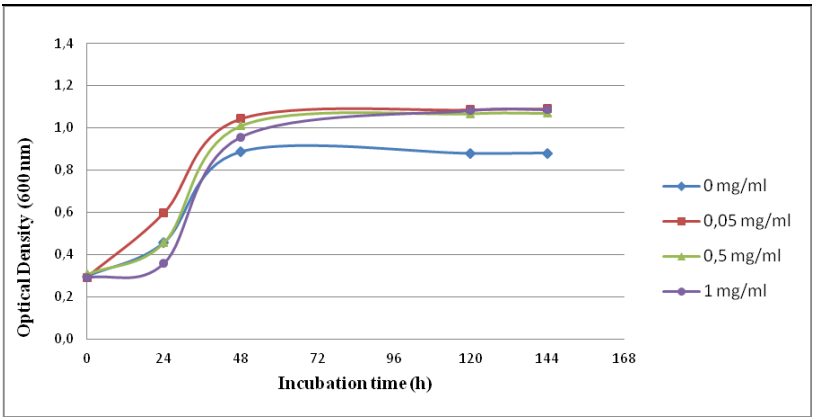


Fig.4.21: Growth curve of CONT-Z (*Rhodococcus sp.*) in presence of various bio-Z concentration (0,05-0,5-1 mg/ml).

Finally, the presence of *Bio-Z* clearly favors the growth of the strain TPBS 9 (*Paenibacillus sp*.) at increasing concentrations (fig. 4.22).

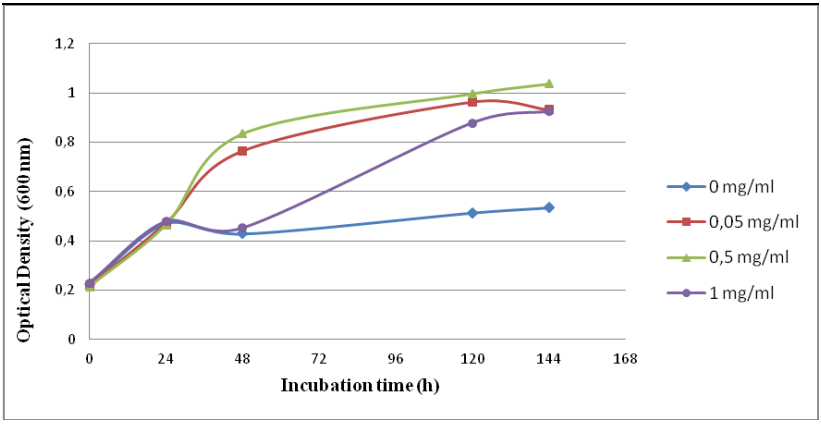


Fig.4.22: Growth curve of TPBS 9 (*Paenibacillus sp.*) in presence of various bio-Z concentration (0,05-0,5-1 mg/ml).

4.5 Cleaning trials on lab-made samples

In order to assess the SACs ability to remove fatty and fume deposits, cleaning trials were performed on brick samples prepared in laboratory, as described in cap. 3.7.

The application of *Bio-Z* on the surface of the brick samples was carried out by a laponite gel added with the SACs solution at 2 mg/ml, according to the procedure described in cap.3.7. A gel of laponite in water was applied as a negative control. After 2, 4 and 6 hours, the gel was removed and the efficacy of the treatment was evaluated.

In regard to the cleaning of the bricks with fume deposits, a whitening of the surface was observed at the naked eye, already visible after 2 hours and more evident after 6 hours of treatment (fig. 4.23). As shown in fig.4.24, the cleaning operated by *Bio-Z* gel proved to be more effective than the action of the control.

Fig.4.23: A brick with fume deposits, before (left) and after (right) the treatment; BIO-Z= laponite + *Bio-Z*; C-= negative control. The gel was removed after 2 (2h), 4 (4h) and 6 (6h) hours of application.

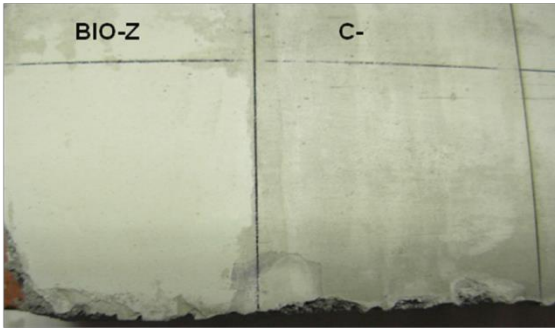


Fig.4.24: Image detail of the brick with fume deposits, after 6 hours of treatment with *BIO-Z* in laponite gel and with laponite gel, as negative control (C-).

Regarding the fat deposits bio-removal, the cleaning performance of *Bio-Z* can be observed only under UV light, due to the fluorescent nature of the fat deposits, not detectablee under visible light.

From the comparison between the UV images before and after the treatment (fig. 4.25), the application of *Bio-Z* turned out to be effective in removing olive oil deposits.

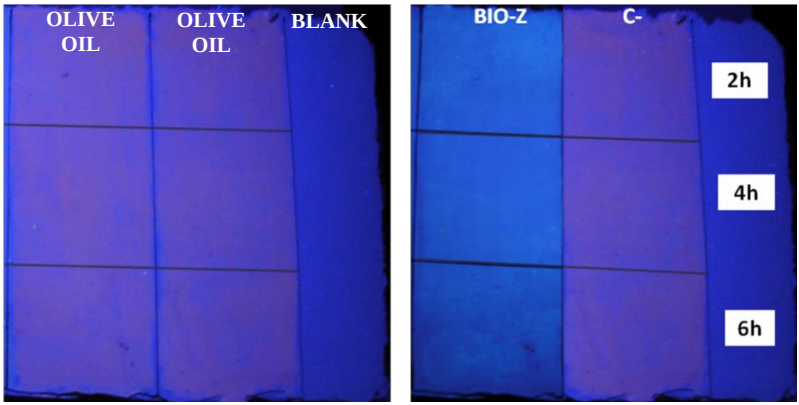


Fig.4.25: A brick sample covered with OLIVE OIL before (left) and after (right) 2, 4 and 6 hours of treatment with *BIO-Z* in laponite gel and laponite gel, (negative control C-) . BLANK represents the plaster without deposits.

In fact, *Bio-Z* determined a greater reduction in fluorescence compared to the negative control, indicating an efficient removal of

the olive oil deposit from the surface of the plaster, reaching its best performance after just 2 hours.

On the contrary, *Bio-Z* was not effective in the removal of animal fat deposits (see fig. 4.26).

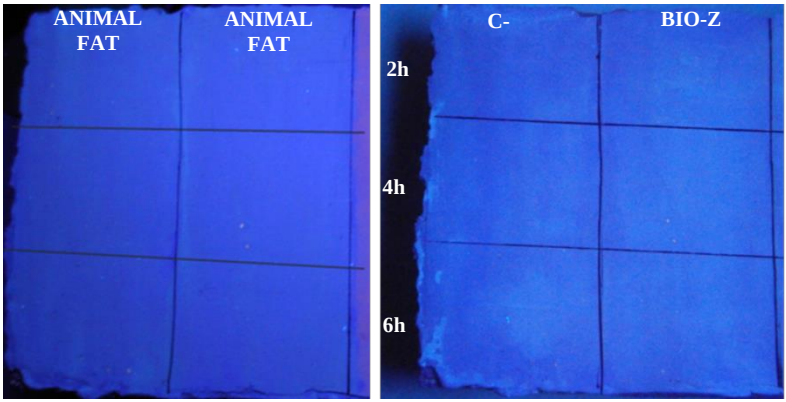


Fig.4.26: A brick specimen covered with ANIMAL FAT before (left) and after (right) 2, 4 and 6 hours of treatment with *BIO-Z* and laponite gel. (negative control C-).

The removal efficacy of olive oil and animal fat deposits, was evaluated also by colorimetric measurements, carried out before and after the treatment.

The values of L*a*b* are reported in the table 4.3, before and after 6 hours of treatment with laponite added or not with *Bio-Z*, as the mean of 3 determinations.

Table 4.3: Mean of the value of L*a*b* parameters			
	L*	**a***	**b***
Blank	**86,48±0,24**	**3,99±0,21**	**90,06±0,20**
Olive oil non-treated	**80,86±0,78**	**4,79±0,19**	**89,64±0,48**
Animal fat non-treated	**89,28±0,94**	**3,99±0,20**	**89,71±0,63**
Olive oil treated with *Bio-Z*	**85,36±0,90**	**4,34±0,24**	**89,83±0,45**
Aniaml fat treated with *Bio-Z*	**89,00±0,43**	**4,01±0,24**	**89,88±0,33**
Olive oil treated with laponite (C-)	**80,92±0,65**	**4,44±0,12**	**89,70±0,44**
Animal fat treated with laponite (C-)	**89,33±0,46**	**4,03±0,25**	**89,70±0,33**

Observing the bar-graph in figure 4.27, the overall difference between the colorimetric values of the blank and those of the surface with olive oil deposits before treatment ($\Delta E^*=5.69$) was mainly at the expense of the L* component, whose decrease ($\Delta L^*=5.62$) indicates a reduction in the brightness of the surface due to the

presence of olive oil. After six hours of treatment with *Bio-Z* , the difference between L* values of blank and olive oil area significantly decreased (ΔL*=1.20), with a consequent improvement in terms of brightness. On the contrary, the application of laponite without *Bio-Z* (negative control) did not decrease the L* value.

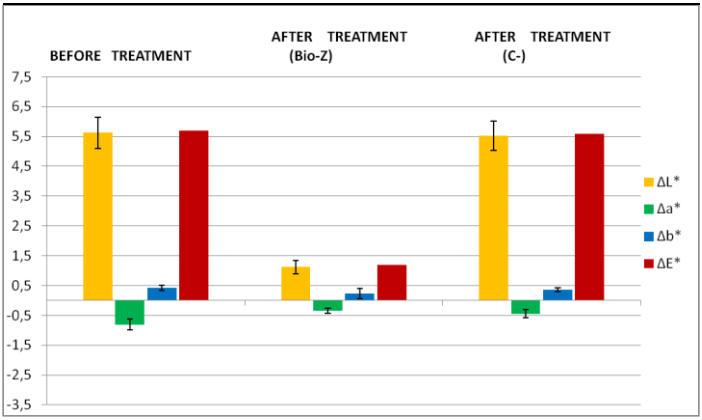


Fig.4.27: ΔL*, Δa*, Δb* and ΔE* values (between Blank area and olive oil spread areas) BEFORE treatment and AFTER 6h of TREATMENT with laponite plus *Bio-Z* (BIO-Z) or laponite in water (C-). The reported values are the mean of 3 determinations.

Regarding the animal fat deposits, the bar-graph in figure 4.28 does not describe a significant variation of the colorimetric parameters. The presence of animal fat determines just a little increase of the L* coordinate. After treatments with laponite plus

Bio-Z and laponite in water (C-) no significative changes were observed in the colorimetric coordinates.

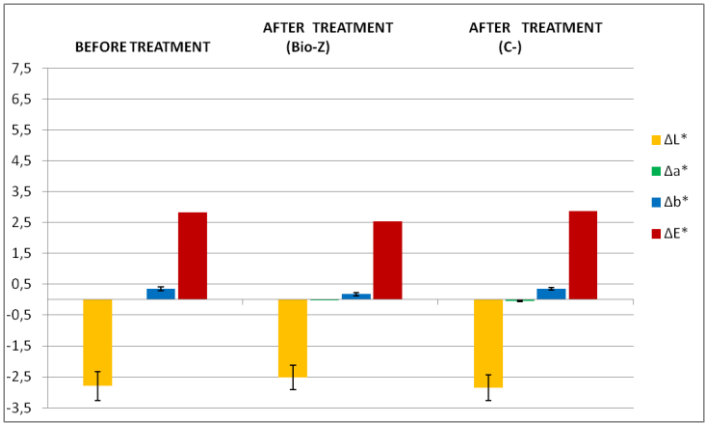


Fig.4.28: ΔL*, Δa*, Δb* and ΔE* values (between Blank area and animal fat spread areas) BEFORE treatment and AFTER 6h of TREATMENT with laponite plus *Bio-Z* (BIO-Z) or laponite in water (C-). The reported values are the mean of 3 determinations.

In order to further evaluate the removal efficacy of *Bio-Z*, the the brick was stained with Sudan III, a dye which selectively binds lipids. Results of staining confirm that the treatment was effective for olive oil removal, while was slightly effective for animal fat removal. showing a different colour gradation among the areas of the specimen (Fig. 4.29).

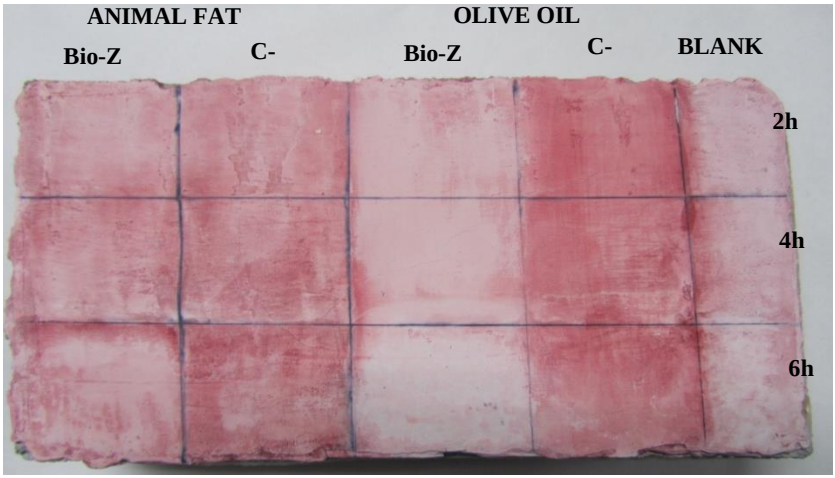


Fig.4.29: Sudan III staining of brick surface after 2, 4 and 6 hours of treatment with laponite plus *BIO-Z* or laponite in water.

4.6 Bio-cleaning trials on the fresco "*The story of Hercules and Cacus*" at Casina Farnese, Palatino site

As part of the restoration in progress at Casina Farnese the original *fresco* of the lodge (fig. 4.30), which shows the killing of Cacus by Hercules, is deteriorated by black deposits. *Bio-Z* was used for preliminary cleaning trials, in the parts were the chemical products usually used were not effective.

The treatment was aimed to remove the thick and complex layer of casein and smog deposits, which made the *fresco* unreadable.

According to the procedure tested in the laboratory trials, as described in cap. 3.9, the *Bio-Z* laponite gel was applied on the selected area of the *fresco* (fig. 4.30) and removed after 18 hours. Figures 4.32-4.32 show the results on selected areas before and after the treatment with *Bio-Z.*

Fig.4.30: Casina Farnese: the lower loggia's fresco "*The story of Hercules and Cacus*", before treatment. The yellow box indicates the area chosen for the trial.

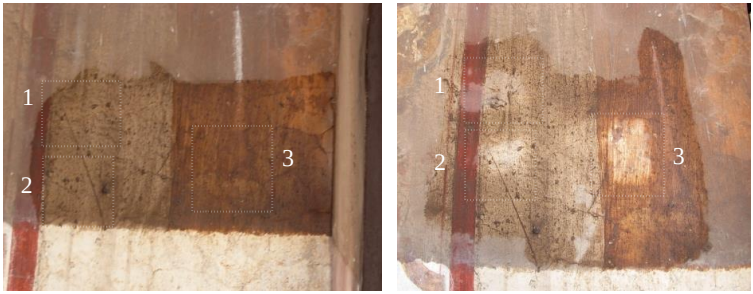


Fig.4.31: Areas of the fresco before (left) and after (right) the treatment: laponite gel as negative control (1), *BIO-Z* laponite gel (2-3). The surfaces looked wet due to the washing step performed before and after the gel application.

Fig.4.32: Treated areas of the fresco as they appeared after drying; negative control (1), treatment (2-3).

Comparing the images before and after the treatment (Fig. 4.31 and 4.32) it is possible to evaluate that , although preliminary, these cleaning trials show the efficacy of the bacterial product *Bio-Z* in removing the deposits layer and making the original colors of the *fresco* emerging.

5. CHAPTER
DISCUSSION AND CONCLUSIONS

Biorestoration of Cultural Heritage by means of biotechnology (bacteria or their metabolic products) is a field of scientific research applied to conservation, which is still in its experimental phase. It is only in the last decade that it has generated increasing interest due to the promising results it obtains. Biotechnology not only offers a useful tool in the study of microorganisms as a cause of deterioration, but it can also provide interesting solutions to the problems of conservation and restoration (Rinaldi 2006). Only a minority of known microorganisms actually play a negative role in the deterioration of artworks, while the vast majority are responsible for "virtuous processes."

Microbiology and biotechnology are still little used in the field of conservation of Cultural Heritage materials (Rinaldi 2005), but the microbial potential for effective treatments of deteriorated artworks is gradually being confirmed and promising results in

connection with this methodology have been obtained (Ranalli *et al*. 2005; Antonioli *et al*. 2005; Cappitelli *et al*. 2010).

The studies currently being pursued aim to expand the knowledge and the range of microorganisms and microbial products available for potential use in biorestoration. Attention is now being focused on bio-molecules with surfactant properties, which might be employed at a delicate stage of cleaning the surfaces of artworks, with a view to finding a suitable alternative to conventional methods when they are considered invalid or toxic to restorers and artworks.

The present work is subdivided into three phases.

The **first part** of the work was aimed at identifying novel bacterial strains able to produce biopolymers with strong superficial and interfacial properties (Surface Active Compounds, SACs). The preliminary screening was carried out using three effective and rapid tests sets, in accordance with trials already described in previous scientific papers, to select the best biosurfactant-producers among the tested microorganisms.

The Oil Spreading Test and Drop-collapse Trial (Techaoei *et al*. 2007; Jain *et al*. 1991) allow for a rapid and cheap qualitative evaluation of the surface properties of produced biosurfactants. Additionally, the Emulsification Test (E_{24}) (Cooper & Goldenberg 1987) gives clear information on emulsifying capacity.

Strains of the type *Pseudomonas aeruginosa* and *Bacillus subtilis* were used as positive controls, in accordance with trials

reported in earlier scientific papers (Rahman *et al*. 2002; Kyung – Taek Oh *et al*. 2006; Kumar *et al*. 2008; Mohammad *et al*. 2008; Priya & Usharani 2009). The bacterial strains were tested in two different culture media, described in the literature for the production of rhamnolipids (by *Ps. aeruginosa* – Zhang-Miller 1992) and surfactin (by *B. subtilis* – Cooper 1981).

Several studies have shown that the type, quality and quantity of biosurfactants produced are influenced by several factors: the composition of the culture medium, the nature of carbon substrate, the concentration of nitrogen, phosphorous, magnesium, ferric and manganese ions and the culture conditions, such as pH, temperature, agitation and dilution rates in continues cultures.

The selection of the microbial strains to be subjected to the screening was done mainly in order to meet the following criteria:

the need to provide procedures that were without risk to either human health or the artwork. This need resulted in the exclusion of both pathogenic and spore-forming microorganisms. The exclusion of spore-forming strains was necessary in order to avoid the presence of dormant spores, which could remain in a persistent form and germinate after the treatment;

the strains used were specifically selected from our ENEA laboratory collection, consisting of strains isolated from

environmental and archaeological sites and characterized by specific metabolic functions.

Based on the results of the screening (tab. 4.1), MCC-Z, *Pedobacter sp*., was selected as the best performing strain, producing molecules with good surface properties (diameter of drop-collapse of 4 mm) and emulsification activity (E_{24}= 54-56%). MCC-Z (fig. 4.3) belongs to *Sphingobacteriaceae* family and *Pedobacter* genus (Steyn *et al*. 1998), a genus not yet described as a bioemulsifier producer and not yet used in the restoration field. Within the same family, *Sphingobacteruim detergens* was described as a biosurfactant producer by Marqués *et al*. in 2012.

This research was followed by the study of the kinetics of the growth of the bacterial strain MCC-Z and the kinetics of the biosurfactant production in order to optimize the production process. The SAC production was evaluated, through the test E_{24}, by growing MCCZ in a mineral medium (MSM, described by Bodour *et al*. 2004) in the presence of different percentages of glucose, added as sole carbon source (fig. 4.4). These series of experiments identified 96 hours as the incubation time for SAC production (fig. 4.5 - 4.6), corresponding to the stationary phase of the growth, and 0.5% (w/v) the optimal concentration of glucose (E_{24} = 64%). An excellent result in view of reducing the cost of raw materials. Low glucose concentration for an optimal production seems to meet the hypothesis that the production by synthesis of these bio-molecules

can be part of a survival strategy of microorganisms in the presence of unfavorable growing conditions (Mukherjee & Das 2005).

Another major factor is the localization of bio-molecules with respect to the bacterial cell. The E_{24} values measured for the whole MCC-Z culture and for the centrifuged and filtered culture (to remove bacterial residues) lead to the conclusion that the biosurfactant is extra-cellular (fig. 4.7), then released into the medium. This allows us to use the bio molecules, separating them easily from the bacterial cells.

The optimal procedure for the SAC extraction was then studied producing a lyophilized crude extract, called *Bio-Z*, that retains its emulsifying power, as demonstrated by repeated tests E_{24} (E_{24} = 62-64%).

In the **second part** of the research, the physico-chemical characterization of *Bio-Z* was carried out. A preliminary analysis, carried out by the means of biochemical assays, revealed that the crude extract *Bio-Z* is composed mainly of carbohydrates, 98% (by weight) with the remaining percentage being composed of a protein fraction. We can assume, therefore, that *Bio-Z* is mainly composed of glycoproteins.

The measurements of surface tension showed that *Bio-Z* reduces the surface tension to about 40 mN/m (fig. 4.13), demonstrating a good surfactant action.

The study of the stability of *Bio-Z* showed that the bio-polymer generates a stable emulsion over time, with E_{24} values remaining almost constant for up to 4 months (fig. 4.12). Also the variation of several parameters, such as temperature, pH and salinity did not alter the emulsifying capacity (fig. 4.9 – 4.10 – 4.11). The stability demonstrated by *Bio-Z*, allows for a wide variation in the application conditions, representing undoubtedly, a positive factor.

The ability of *Bio-Z* to effectively emulsify a series of hydrocarbons has been tested (tab. 4.2). Hexadecane, toluene, xylene, isooctane, cyclohexane and diesel represent common environmental contaminants and are often included in the artistic surface deposits.

There are numerous studies of bioremediation in the environmental field in which the biosurfactants promote the biodegradation of hydrocarbons (Zhang & Miller 1995; Kosaric 2001; Saeki *et al*. 2009). Based on some positive results obtained in the environmental sector, particularly in applications of bioremediation of polluted soils and waters (Banat *et al*. 2000; Malik Haddadin *et al*. 2009), we decided to experiment with a transfer of these technologies to the field of Cultural Heritage. In fact, the combustion of crude oil (originating from various human activities) produces a wide range of pollutants, including gases, volatile organic compounds, polycyclic aromatic hydrocarbons, acid compounds and soot. Several of these pollutants have been linked with the

deterioration and blackening of monuments (Bonazza *et al*. 2007). So, the use of biosurfactants could facilitate the removal of the undesirable deposits of smog and soot particles, which often cover the surface of outdoor artworks.

Among the many properties described in the literature for biosurfactants, the anti-microbial activity (Vollenbroich *et al*. 1997; Mukherjee *et al*. 2006; Rivardo *et al*. 2009), studied particularly in the medical field (Banat *et al*. 2010), could have an interesting application in the field of conservation of Cultural Heritage. Rodrigues *et al*. (2006) speculate that the biosurfactants might contain signaling factors that interact with the host and/or bacterial cells, leading to the inhibition of infections. Moreover, they support the assertion of a possible role in preventing microbial adhesion and, as a result, their growth on the different substrates.

For this purpose, eight bacterial strains (tab. 3.2), among the microorganisms described in scientific papers as responsible for different phenomena in the bio-deterioration of Cultural Heritage, were chosen. In particular, we used *Bacillus*, isolated from an archeological site, *Pseudomonas*, (Altenburger *et al*. 1996; Heyrmanm *et al*. 1999, 2006), *Rhodococcus* (Groth & Saiz-Jimenez 1999) and *Acinetobacter* genera (Palla 2005), isolated from contaminated sites, to evaluate the anti-microbial activity of *Bio-Z*. The different response of the tested bacterial strains to *Bio-Z* showed the presence of anti-microbial activity even at very low

concentrations (0.05% w/v) of the biosurfactant, but the level of response depended on the microbial species tested. According to others authors data (Satirova *et al*. 2008; Benincasa *et al* 2004), *Bio-Z* has an inhibitory effect on Gram-positive strains (TPBF 11-*Cellulomonas sp*., TPBF 14-*Bacillus cereus*, DAN 10-*Bacillus pumilus* and DPBS 17-*Bacillus megaterium* / fig.4.15 – 4.16 – 4.17 - 18) but not on Gram-negative strains (LAM 21-*Acinetobacter calcoaceticus* / fig. 4.19). In our case the strains belonging to *Rhodococcus* and *Paenibacillus* genera, although Gram-positive, are not influenced by the presence of *Bio-Z* (fig. 4.20 – 4.21 – 4.22). Therefore, the assessment of the inhibitory action on bacterial growth by *Bio-Z* could be useful with a view to the use of the bio-product for the control of biodeterioration.

In the **third and final part**, *Bio-Z* was used for cleaning tests, first on laboratory samples and then for *in vivo* applications. In the laboratory, we prepared different types of plastered bricks. In the first case, the brick had been blackened with fumes from burning oil, fat and kerosene (fig. 3.4); in the second case, it was covered with fatty and oily deposits (fig. 3.3). The bricks were then aged artificially by thermal treatment in order to simulate the transformations that occur and the various kinds of deposits that develop over time on mural paintings and which are difficult to remove with chemical surfactants, even when used in combination with mechanical methods.

The application of *Bio-Z* on the plaster was conducted using laponite gel. The use of gel as an agent in cleaning operations is very widespread in restoration because it allows for the removal of the undesired product, limiting the release of water that could cause mechanical or physical stresses on the treated substrate.

In order to evaluate the effectiveness of cleaning, the color changes after treatment were analyzed by means of visual inspection under visible and UV light, by colorimetric measurements and staining with selective dyes. The observations made under visible light have highlighted the cleaning action of *Bio-Z* on the layer of *nerofumo* on plaster (fig. 4.23 – 4.24). As regards the brick with fatty deposits, the observations made under ultraviolet clearly showed a reduction of the fluorescence emitted by the layer of fat following the treatment with *Bio-Z*, in comparison with the negative control done using only laponite and water (4.25). The analysis of the colorimetric parameters showed that the presence of fatty deposits, before treatment, resulted in a change in color compared to Blank, a clean brick devoid of deposits. On the portion characterized by deposits of vegetable oils, the difference compared to Blank is reduced after treatment with *Bio-Z* with an improvement in terms of brightness (parameter L*), which is not visible after treatment with the negative control (fig. 4.27). The colorimetric analysis conducted on the portion of deposits with animal fat does not allow a proper assessment because animal fat produces a whitening of the plaster

and the action with *Bio-Z* does not produce significant changes from the negative control (fig. 4.28).

A last evaluation of the *Bio-Z* cleaning activity was carried out by staining lipids with Sudan III. This dye allowed us to confirm what was seen by the colorimetric analysis (fig. 4.29). In the portion of vegetable oil deposits, the coloring was much less intense in the portion treated with *Bio-Z*, equal to Blank devoid of any deposit, compared to the negative control. As regards the animal fat deposit, coloring between *Bio-Z* and the negative control was almost identical. The cleaning tests on specimens allowed us to confirm, firstly, the cleaning action of *Bio-Z*, secondly, at the same time its non-aggressive nature towards the delicate materials with which it came into contact, and, thirdly, its differing performance dependent on the nature of the substrate to which it was applied. The different chemical composition, in fact, between deposits of plant origin (mixtures of mono unsaturated fats) and those of animal origin (consisting of saturated fatty acids), which undergo various chemical changes due to ageing, might explain the differential action of *Bio-Z* to remove surface deposits. This is an important characteristic in view of the use of *Bio-Z* as a new product in the restoration field with a view to differential cleaning of artistic surfaces.

Based on the promising results obtained on the specimens, the work continued with *Bio-Z* application *in vivo*, on the frescoes in

the Casina Farnese loggia, characterized by deposits of caseinates combined with smog and carbonaceous particles (fig. 4.30).

The use of the traditional cleaning technique on the surface of the fresco proved inefficient. So the restorers were looking for an alternative method of cleaning, based on the use of novel products, such as *Bio-Z.*

Treatments with ammonium carbonate poultices and with a scalpel were ineffective in cleaning the frescoes in the lower loggia, while the use of *Bio-Z,* supported by laponite gel, facilitated the removal of surface deposits and restored the original color of the plaster (fig. 4.31 – 4.32). The treatments involved in this research are by way of being preliminary trials.

Following the beneficial results of the first tests, we have carried out other tests, which are still in progress, and are being conducted using *Bio-Z* alone or in combination with bacterial strains. With complementary properties. The microorganism were selected according to the substrate to be removed.

Although the establishment of a new procedure of bioremediation by means of *Bio*-Z will require refinements which have not been fully explored in the current project and further development, we can say that this research has already achieved important goals, compared to the current procedures available:

- the selection of the strain, MCC-Z *Pedobacter sp.*, never previously described in scientific papers either as a producer of biosurfactants or for bio-restoration applications;

- the obtaining of a bio-product, *Bio-Z*, with good surfactant characteristics (bringing about a reduction in surface tension of about 40 mN/m), emulsifying capacity, good stability over time and resistant to changes in pH, temperature and salinity;

- tests on laboratory specimens and testing on the *in vivo* clean up of Casina Farnese frescoes have shown that *Bio-Z* can be configured as a new product of microbial origin, efficient and selective, for use in bio-cleaning of historical and artistic surfaces;

- the anti-microbial properties of *Bio-Z* confirms its value in bio-restoration and also as a control product against biodeterioration.

Through extensive study and a controlled use of microorganisms (or their derivates) as bioremediation agents, it will be possible to provide a valuable support to conservation and restoration interventions. This approach has several advantages: it is effective, not destructive and capable of removing only the extraneous substances from the artwork, in comparison with traditional chemical-physical methods, often aggressive. Moreover another advantage is the use of non-toxic microorganisms, which are safe for both the operators and the environment, as well as for the artwork. Studies conducted to date highlight that the contribution of the microbial world in the preservation of artworks is being

comprehensively investigated and considered, but that an optimal solution does not rely solely on biological methods. It requires strong interaction and integration with physical and chemical based methods, as well as a better understanding and dialogue between the scientific-technological world and the art world (Fernandes 2006).

Lastly, this paper sets out an alternative approach to old problems, through the introduction of new products for cleaning damaged artistic surfaces. We want to provide the foundation for an innovative and considered choice in restoration methods, a choice which leads to restoration projects that are both functional and well targeted, not only in the view of the much publicized scientific approach to restoration, but above all from the point of view of awareness and sustainability.

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REFERENCES

- Abdel-Mawgoud A.M., Aboulwafa M.M., Abdel-Haleem Hassouna N. (2008). Characterization of Surfactin Produced by *Bacillus subtilis* Isolate BS5. In *Applied Biochemistry and Biotechnology* 150 (3): 289-303.

- Abdel-Mawgoud A.M., Aboulwafa M.M., Abdel-Haleem Hassouna N. (2009). Characterization of Rhamnolipid Produced by *Pseudomonas aeruginosa* Isolate Bs20. In *Applied Biochemistry and Biotechnology* 157 (2): 329-345.

- Abouseoud M., Maachi R., Amrane A., Boudergua S. and Nabi A. (2008). Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*. In *Desalination* 223: 143-151.

- Adolphe, J.M., Loubière J.F., Paradas J. and Soleilhavoup F. (1990). Procédé de traitement biologique d'une surface artificielle. European patent 90400G97.0 (after French patent 8903517, 1989).

- Al-Araji L., Rahman R.N.Z.R.A., Basri M., Salleh A.B. (2007). Microbial Surfactant- MINIREVIEW. In *Asia Pacific*

Journal of Molecular Biology and Biotechnology 15 (3): 99-105.

- Antonioli P., Zapparoli G., Abbruscato P., Sorlini C., Ranalli G. and Righetti P.G. (2005). Art-loving bugs: The resurrection of Spinello Aretino from Pisa's cemetery. In *Proteomics* 5: 2453-2459.

- Arauz L.J., Jozala A.F., Mazzola P.G. and Penna T.C.V. (2009). Nisin biotechnological production and application: A review. In *Trends Food Sci. Technol*. 20: 146-154.

- Asci Y., Nurbas M. and Acikel Y.S. (2010). Investigation of sorption/desorption equilibria of heavy metals ions on/from quartz using rhamnolipid biosurfactant. In *J. Environ. Manage*. 91: 724-731.

- Atlas R.M. (1995). Bioremediation. Chem. Eng. News 73, 32-42.

- Baglioni P. and Giorgi R. (2000). Metodologie chimico-fisiche innovative per la conservazione dei dipinti murali. In *OPD Restauro*, Firenze 2000, 11: 76-84.

- Banat I. M., Franzetti A., Gandolfi I., Bestetti G., Martinotti M.G., Fracchia L., Smyth T.J. and Marchant R. (2010). Microbial biosurfactants production, applications and future

potential. In *Applied Microbiology and Biotechnology* 87: 427-444.

- Banat I.M., Makkar R.S., Cameotra S.S. (2000). Potential commercial applications of microbial surfactants. In *Applied Microbiology and Biotechnology* 53: 495-508.

- Bang Sookie S., Galinat Johnna K. and Ramakrishnan V. (2001). Calcite precipitation induced by polyurethane-immobilized *Bacillus pasteurii*. In *Enzyme and Microbial Technology* 28: 404–409

- Banik, G. (2003). Removal of starch paste adhesives and relinings from paper-based objects by means of enzyme poultices /, In: *Materiali tradizionali ed innovativi nella pulitura dei dipinti e delle opere policrome mobili: atti del convegno, Piazzola su Brenta (PD), 25-26 ottobre 2002* / Cremonesi, Paolo (*ed*); Padova: Il Prato: 33-38.

- Barabesi C., Mastromei G. and Perito B. (2006). La carbonatogenesi batterica nella conservazione delle opere d'arte lapidee; in *Biologia e archeobiologia nei beni culturali, conoscenze, problematiche e casi di studio,* AIAR e Musei Civici Como editore: 236 – 247.

- Barabesi, C., Galazzi, A., Mastromei, G., Rossi, E., Tamburini, E. and Perito B. (2007). Bacillus subtilis gene cluster involved in calcium carbonate biomineralization. In *Journal of Bacteriology* 189: 228-235.

- Bellucci R. and Cremonesi P. (1994). L'uso degli enzimi nella conservazione e nel restauro dei dipinti. In: *Kermes: arte e tecnica del restauro* 7 (21): 45-64.

- Benincasa M., Abalos A., Oliveira I., Manresa A. (2004). Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock. In *Antonie van Leeuwenhoek* 85: 1-8.

- Blüher, A., Banik, G., Thobois, E. (1996). The application of Carbopol gels and enzyme containing methylcellulose gels for removal of starch based adhesives in albums. In *International conference on Conservation and Restoration of archive and library* materials, Erice (Italy), CCSEM, 22-29 April 1996: vol. I and II.

- Bodour A.A. & Mille-Maier R.M. (1998). Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms. In *Journal of Microbiological Methods* 32 (3): 273–280.

- Bodour A.A., Drees K.P. and Maier R.M. (2003). Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. In *Applied and Environmental Microbiology* 69 (6): 3280-3287.

- Bodour A.A., Guerrero-Barajas C., Jiorle B.V., Malcomson M.E., Paull A.K., Somogyi A., Trinh L.N., Bates R.B., Maier R.M. (2004). Structure and Characterization of Flavolipids, a Novel Class f Biosurfactants Produced by Flavobacterium sp. Strain MTN11. In *Applied and Environmental Microbiology* 70 (1): 114-120.

- Bonaccini E. and Cremonesi P. (2001). Preparazione e utilizzo di gel chelanti, reagenti per la pulitura di opere policrome. In *Progetto restauro*, Anno 8, N. 19: 11-21.

- Bonazza A., Sabbioni C., Ghedini N., Hermosin B., Jurado V., Gonzalez J.M. and Saiz-Jimenez C. (2007). Did smoke from the Kuwait oil well fires affect iranian Archaeological Heritage? In *Environ. Sci. Technol*. 41: 2378-2386.

- Bonini M., Lenz S., Falletta E., Ridi F., Carretti E., Fratini E., Wiedenmann A. and Baglioni P. (2008). Acrylamide-Based Magnetic Nanosponges: A New Smart Nanocomposite Material Langmuir 24: 12644-12650.

- Bonomi R. (1994). Utilizzo degli enzimi per il restauro di una scultura in terracotta policroma, in *OPD Restauro* 6:101-107

- Bonsanti, G. (2003). Storia ed etica della pulitura. In Congresso internazionale, colore e conservazione: materiali e metodi nel restauro delle opere policrome mobili. Piazzola sul Brenta, Italy. Il Prato, Padova, Italy.

- Bradford M.M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. In *ANALYTICAL BIOCHEMISTRY* 72: 248-254.

- Brusseau M.L., Miller M.L., Zhang Y., Wang X. and Bai G.Y. (1995). Biosurfactant- and cosolvent-enhanced remediation of contaminated media. In *Microbial Processes for Remediation* ed. Hinchee R.E., Brockman F.J.and Vogel C.M., pp. 82-94. Columbus, Ohio: Battelle Press.

- Burgos-Dìaz ., Pons R., Espuny M.J., Aranda F.J., Teruel J.A., Manresa A., Ortiz A. and Marqués A.M. (2011). Isolation and partial characterization of a biosurfactant mixture produced by *Sphingobacterium* sp. isolated from soil. In *Journal of Colloid and Interface Science* 361: 195-204.

- Buttazzoni N., Casoli A., Cremonesi P. and Rossi Paolo (2001). Preparazione e utilizzo di gel enzimatici, reagenti per la pulitura di opere policrome. In Salone dell'arte del restauro

e della conservazione dei Beni Culturali e Ambientali, VIII Ed., FerraraFiere.

- Cacchio P., Ercole C., Cappuccio G. and Lepidi A. (2003). Calcium Carbonate precipitation by bacterial strains isolated from a limestone cave and from a loamy soil. In *Geomicrobiology Journal* 20: 85-98.

- Caley T. (1990). Aspects of Varnishes and the Cleaning of oil Paintings Before 1700. In Cleaning, Retouching and Coatings, ed. Mills, J. S, and Smith P., London: International Institute for Conservation of Historic and Artistic Works: 70-72.

- Cappitelli F., Toniolo L., Sansonetti A., Gulotta D., Ranalli G., Zanardini E. and Sorlini C. (2007). Advantages of using microbial technology over traditional chemical technology in removal of black crusts from stone surfaces of historical monuments. In *Applied and Environmental Microbiology* 73 (17): 5671–5675.

- Cappitelli F., Zanardini E., Ranalli G., Mello E., Daffonchio D. and Sorlini C. (2006). Improved Methodology for Bioremoval of Black Crusts on Historical Stone Artworks by Use of Sulfate-Reducing Bacteria. In *Applied and Environmental Microbiology* 72 (5): 3733–3737.

- Carretti E., Giorgi R., Berti D. and Baglioni P. (2007). Oil-in-water nanocontainers as low environmental impact cleaning tools for works of art: two case studies. In *Langmuir* 23: 6396-6403.

- Castanier S., Le Metayer-Levrel G. and Perthuisot J.P. (1999). Ca-carbonates precipitation and limestone genesis— the microbiogeologist point of view. In *Sedimentary Geology* 126: 9–23.

- Castanier, S., Le Métayer-Levrel, G. and Loubière, J.F. (1995). Nouvelles compositions pour mortier biologique, procédé de recouvrement d´une surface ou de comblement d´une cavité à l'aide des compositions. French Patent nº 9505861.

- Chen S.Y., Wei Y.H. and Chang J.S. (2007). Repeated pH-stat fed-batch fermentation for rhamnolipid production with indigenous Pseudomonas aeruginosa S2. In *Applied Microbiology and Biotechnology* 76 (1): 67-74.

- Christofi N. and Ivshina I.B. (2002). Microbial surfactants and their use in field studies of soil remediation. In *Journal of Applied Microbiology* 93: 915-929.

- Christofi N. and Ivshina I.B. (2002). Microbial surfactants and their use in field studies of soil remediation. In *Journal of Applied Microbiology* 93: 915–929.

- Cooper D.G. & Goldenberg B.G. (1987). Surface-Active Agents From Two Bacillus Species. In *Applied Environmental Microbiology*, February, 53(2): 224-229.

- Cooper D.G., MacDonald C.R:, Duff S.J.B. and Kosaric N. (1981). Enhanced Production of Surfactin from Bacillus subtilis by Continuous Product Removal and Metal Cation Additions. In *Applied and Environmental Microbiology*, September, 42: 408-412.

- Cremonesi P. (1997). Materiali e metodi per la pulitura di opere policrome, Phase – Firenze.

- Cremonesi P. (1999). L'uso degli enzimi nella pulitura di opere policrome. Padova, Italy : Il Prato, p 96.- I talenti 4: metodologie, tecniche e formazione nel mondo del restauro.

- Cremonesi P. (2000). L'uso dei solventi organici nella pulitura di opere policrome. I Talenti – Metodologie, tecniche e formazione nel mondo del restauro, Il Prato, Padova.

- Cremonesi P. (2004). L'uso dei solventi organici nella pulitura di opere policrome. Padova, Italy : Il Prato, p. 166.-I talenti 7: metodologie, tecniche e formazione nel mondo del restauro.

- Cubitto M.A., Mora´ n, A.C., Commendatore, M., Chiarello, M.N., Baldini, M.D., Sin˜ eriz, F. (2004). Effects of *Bacillus subtilis* O9 biosurfactant on the bioremediation of crude oil-polluted soils. In *Biodegradation* 15: 281–287.

- Cutler S.R., Rodriguez P.L., Finkelstein R.R., Abrams S.R. (2010). Abscisic acid: Emergence of a core signaling network. In *Annu Rev Plant Biol* 61*:* 651–679.

- Das P., Mukherjee A.K. and R. Sen (2009). Antiadhesive action of a marine microbial surfactant. In *Colloids Surf B: Biointerfaces* 71: 183-186.

- Daverey A. and Pakshirajan K. (2010). Kinetics of Growth and Enhanced Sophorolipids Production by *Candida bombicola* Using a Low-Cost Fermentative Medium. In *Applied Biochemistry and Biotechnology* 160 (7): 2090-2101.

- De Carvalho C.C.C.R. and de Fonseca M.M.R. (2005). Degradation of hydrocarbons and alcohols at different temperatures and salinities by *Rhodococcus erythropolis* DCL14. In *FEMS Microbiology Ecology* 51: 389-399.

- De la Chapelle A. (1990). Décollage enzymatique de dessins doubles. Une approche expérimentale. / 2 vols. Memoire de fin d'etude, Institut Français de Restauration des Oeuvres d'Art.

- De La Chapelle A. (2003). Utilizzo degli enzimi nel restauro delle opere grafiche policrome /, In: *Materiali tradizionali ed innovativi nella pulitura dei dipinti e delle opere policrome mobili: atti del convegno, Piazzola su Brenta (PD), 25-26 ottobre 2002* / Cremonesi, Paolo (*ed*); Padova: Il Prato: 51-53.

- Dehghan-Noudeh G., Housaindokht M. and Bazzaz B.S.F. (2005). Isolation, characterization and investigation of surface and hemolytic activities of a lipopeptide biosurfactant produced by *Bacillus subtilis* ATCC 6633. In *J. Microbiol*., 43: 272-276.

- Desai J.D. and Banat I.M. (1997). Microbial production of surfactants and their commercial potential. In *Microbiol. Mol. Biol. Rev*. 61: 47-64.

- Deziel E., Pauette G., Villemur R., Lepine F. and Bisaillon J.G. (1996). Biosurfactant Production by a Soil Pseudomonas Strain Growing on Polycyclic Aromatic Hydrocarbons. In *Applied And Environmental Microbiology* 62 (6): 1908-1912.

- Dubois M., Gills K.A., Hamilton J.K., Rebers P.A. and Smith F. (1956). Colorimetric method for determination of sugar and related substances. In *Anal. Chem.* 28: 350–356.

- Ehrlich H.L. (2000). *Geomicrobiology:* New York, Marcel Dekker, 719 p.

- Erhardt J. and Bischoff J. (1994). The roles of various components of Resin Soaps, bile acid soaps and gels, and their effects on oil paint films. In *Studies in Conservation* 39: 3-27.

- Fernandes P. (2006). Applied microbiology and biotechnology in the conservation of stone cultural heritage materials. In *Applied Microbiology and Biotechnology* 73: 291-296.

- Fernandes P.A.V., Arruda I.R., Santos A.F.B, Arajo A.A., Maior A.M.S. and Ximenes E.A. (2007). Antimicrobial activity of surfactants produced by *Bacillus subtilis* R14 against multidrug-resistant bacteria. In *Braz J. Microbiol*. 38: 704-709.

- Fiechter A. (1992). Integrated systems for biosurfactant synthesis. In *Pure Applied Chem*. 64: 1739-1743.

- Flagas M.E. and Makris (2009). Probiotic bacteria and biosurfactants for nosocomical infection control: A hypothesis. In *J. Hospital Infection* 71: 301-306.

- Fornaciari C. (1993). La laponite : esperienze di lavoro; in CAB newsletter: Conservazione negli archivi e nelle biblioteche, v. 2 (9): 6-7.

- Franzetti A., Bestetti G., Caredda P., La Colla P. and Tamburini E. (2008). Surface-active compounds and their role in the acess to hydrocarbons in *Gordonia* strains. In *FEMS Microbiol Ecol* 63: 238-248.

- Franzetti A., Caredda P., La Colla P., Pintus M., Tamburini E., Papacchini M. and Bestetti G. (2009). Cultural factors affecting biosurfactant production by *Gordonia* sp. BS29. In *International Biodeterioration & Biodegradation* 63: 943-947.

- Gandhimathi R., Kiran G.S., Hema T. A., Selvin J., Raviji T.R., Shanmughapriya S. (2009). Production and characterization of lipopeptide biosurfactant by a sponge-associated marine actinomycetes *Nocardiopsis alba* MSA10. In *Bioprocess and Biosystems Engineering* 32 (6): 825-835.

- Gauri K.L. and Chowdhury A.N. (1988). Experimental studies on conversion of gypsum to calcite by microbes. In *Proceedings of 6th International Congress on Deterioration and Conservation of Stone* (Ciabach, J., ed.), pp. 545–550, Nicholas Copernicus University

- Gautam K.K. and Tiagi V.K. (2006). Microbial surfactants: A review. In *J. Oleo Sci*. 55: 155-166.

- González-Muñoz M.T. (2008). Bacterial biomineralization applied to the protection-consolidation of ornamental stone:current development and perspectives. In *COALITION* No. 15: 15-18.

- Groth I., Vettermann R., Schuetze B., Schumann P. and Saiz-Jimenez C. (1999). Actinomycetes in Karstic caves of northern Spain (Altamira and Tito Bustillo). In *Journal of Microbiological Methods* 36 (1-2): 115-122.

- Gusiatin, Z., Klimiuk E., Pokoj T. and Kulikowaska D. (2009). Biosurfactant using soil remediation highly contaminated with heavy metals. In *New Biotechnol*. 25.

- Haddad N.I.A., Wang J. and Mu B. (2009). Identification of a Biosurfactant Producing Strain: *Bacillus subtilis* HOB2. In *Protein and Peptide Letters* 16 (1): 7-13.

- Haddadin M.S.Y., Abou Arqoub Ansam A., Abu Reesh I., Haddadin J. (2009). Kinetics of hydrocarbon extraction from oil shale using biosurfactant producing bacteria. In *Energy Conversion and Management* 50: 983–990.

- Healy M.G., Devine C.M. and Murphy R. (1996). Microbial production of biosurfactants. In *Resour. Conserv. Recy*. 18: 41-57.

- Heselmeyer K., Fischer U., Krumbein W.E. and Warscheid T. (1991). Application of Desulfovibrio vulgaris for the bioconversion of rock gypsum crust into calcite, *BIOforum* 1-2, 89.

- Heyrman J., Mergaert J., Denys R. and Swings J. (1999). The use of fatty acid methyl ester analysis (FAME) for the identification of heterotrophic bacteria present on three mural paintings showing severe damage by microorganisms. In *FEMS Microbiology Letters* 181: 55-62.

- Hirata Y.S., Ryu M., Oda Y., Igarashi K., Nagatsuka A., Furata T. and Sugiura M. (2009). Novelcharacteristics of sophorolipids, yeast glycolipid biosurfactants, as biodegradable low foaming surfactants. In *J. Biosci. Bioeng*. 108: 142-146.

- Hoffiman D.R., Anderson P.P., Schobert C.M., Gault M.B., Blanford W.J. and Sandrin T.R. (2010). toxicity of *Cadmium cobalt* and copper during naphthalene biodegradation. In *Bioresour. Technol*. 101: 2672-2677.

- Hommel R.K. (1990). Formation and physiological role of biosurfactants produced by hydrocarbon-utilizing microorganisms. In *Biodegradation* 1: 107-119.

- Hörmann B., Müller M. M., Syldatk C. and R. Hausmann (2010). Rhamnolipid production by *Burkholderia plantarii* DSM 9509. In *European Journal of Lipid Science and Technology* 112 (6): 674–680.

- Hultberg M., Bengtsson T. and Liljeroth E. (2010). Late blight on potato is suppressed by the biosurfactant-producing strain *Pseudomonas koreensis* 2.74 and its biosurfactant. In *BioControl* 55 (4): 543-550.

- Iannucelli S. and Sotgiu S. (2009). La pulitura superficiale di opere grafiche a stampa con gel rigidi. In: *Progetto Restauro*, Il Prato, Padova, 49: 15-24.

- Ivshina I.B., Kuyukina M.S., Philp J.C. and Christo N. (1998). Oil desorption from mineral and organic materials using biosurfactant complexes produced by *Rhodococcus* species. In *World J Microbiol Biotechnol* 14: 711–717.

- Jain D.K., Collins-Thompson D.L., Lee H. and Trevors J.T. (1991). A drop-collapsing test for screening surfactant-

producing microorganisms. In *Journal of Microbiological Methods* 13: 271-279.

- Janek T., Łukaszewicz M., Rezanka T. and Krasowska A. (2010). Isolation and characterization of two new lipopeptide biosurfactants produced by *Pseudomonas fluore*scens BD5 isolated from water from the Arctic Archipelago of Svalbard. In *Bioresource Technology* 101 (15): 6118–6123.

- Jennings E.M. and Tanner R.S. (2000). Biosurfactant–Producing bacteria found in contaminated and uncontaminated soils. In *Proceedings of the 2000 Conference on Hazardous Waste Research*, 299-306.

- Joshi S., Bharucha C. and Desai A.J. (2008). Production of biosurfactant and antifungal compound by fermented food isolate *Bacillus subtilis* 20B. In *Bioresour. Technol*. 99: 4603-4608.

- Jroundi F., Fernández-Vivas A., Rodriguez-Navarro C., & Bedmar E.J. and González-Muñoz M.T. (2010). Bioconservation of Deteriorated Monumental Calcarenite Stone and Identification of Bacteria with Carbonatogenic Activity. In *Microbial Ecology* No. 60: 39-54.

- Jroundi F., Gómez-Suaga P., Jimenez-Lopez C., González-Muñoz M.T., Fernandez-Vivas M.A. (2012). Stone-isolated carbonatogenic bacteria as inoculants in bioconsolidation treatments for historical limestone. In Science of The Total Environment 425: 89-98.

- Kamiya N, Inoue M, Goto M, Nakamura N, Naruta Y. Catalytic and structural properties of surfactant-horseradish peroxidase complex in organic media. In *Biotechnol Prog*. 16: 52–58.

- Kavamura V.N. and Esposito E. (2010). Biotechnological strategies applied to the decontamination of soils polluted with heavy metals. In *Biotechnol. Adv*. 28: 61-69.

- Kearns D.B. and Losick R. (2003). Swarming motility in undomesticated Bacillus subtilis. In *Mol. Microbiol*. 49: 581–90.

- Khandekar N. (2001). A survey of the conservation literature relating to the development of aqueous gel cleaning on painted and varnished surfaces. In *Reviews in Conservation* 1-2000: 10-20.

- Kiran G.S., Thomas T.A. and Selvin J. (2010b). Production of a new glycolipid biosurfactant from marine *Nocardiopsis*

lucentensis MSA04 in solid-state cultivation. In *Colloids and Surfaces B: Biointerfaces* 78 (1): 8–16.

- Kiran G.S., Thomas T.A., Selvin J., Sabarathnam B. and Lipton A.P. (2010a). Optimization and characterization of a new lipopeptide biosurfactant produced by marine *Brevibacterium aureum* MSA13 in solid state culture. In *Bioresource Technology* 101 (7): 2389–2396.

- Klein K. and Beutel S (2002). Schädigende Kaseinüberzüge auf Wandmalereien. Teil 1: Innovatives Verfahren zur enzymatischen Abnahme. In: Restauro: Zeitschrift für Kunsttechniken, Restaurierung und Museumsfragen , Jahrgang 108, No.7: 496-501.

- Konishi M., Fukuoka T., Nagahama T., Morita T., Imura T., Kitamoto D. and Hatada Y. (2010). Biosurfactant-producing yeast isolated from *Calyptogena soyoae* (deep-sea cold-seep clam) in the deep sea. In *Journal of Bioscience and Bioengineering* 110 (2): 169–175.

- Konishi M., Imura T., Fukuoka T., Morita T., Kitamoto D. (2007a). A yeast glycolipid biosurfactant, mannosylerythritol lipid, shows high binding affinity towards lectins on a self-assembled monolayer system. In *Biotechnol Lett.* 29: 473–480.

- Konishi M., Morita T., Fukuoha T., Imura T., Kakugawa K., Kitamoto D. (2007b). Production of different types of mannosylerythritol lipids as biosurfactants by the newly isolated yeast strains belonging to the genus Pseudozyma. In *Applied Microbiology and Biotechnology* 75: 521–531.

- Kosaric N. (1992). Biosurfactants in industry. In *Pure Applied Chem*. 64: 1731-1737.

- Kosaric N. (2001). Biosurfactants and their application for soil bioremediation. In *Food Technol. Biotechnol*. 39 (4): 295-304.

- Kyung-Taek Oh, Kang C.-M., Kubo M. and Chung S.-Y. (2006). Culture Condition of Pseudomonas aeruginosa F722 for biosurfactant production. In *Biotechnology and Bioprocess engineering* 11: 471-476.

- Lai C.C., Huang Y.C., Wei Y.H. and Chang J.S. (2009). Biosurfactant-enhanced removal of total petroleum hydrocarbons from contaminated soil. In *J. Hazard Mater*. 167: 609-614.

- Lang S. and Philp J.C. (1998). Surface-active lipids in rhodococci. In *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 74: 59–70.

- Lima T.M.S., Procópio L.C., Brandão F.D., Carvalho A.M.X., Tótola M.R. and Borges A.C. (2011). Biodegradability of bacterial surfactants. In *Biodegradation* 22: 585-592.

- Liu X., Ren B., Chen M., Wang H., Kokare C.R., Zhou X., Wang J., Dai H., Song F., Liu M.,Wang J., S. Wang and Zhang L. (2010). Production and characterization of a group of bioemulsifiers from the marine *Bacillus velezensis* strain H3. In *Applied Microbiology and Biotechnology* 87 (5): 1881-1893.

- Magaudda G. (1994) *Il biodeterioramento dei beni culturali*, Borgia Editore, 17-22.

- Maier RM, Soberón-Chávez G (2000). *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. In *Applied Microbiology and Biotechnology* 54: 625–633.

- Marqués A.M., Burgos-Dìaz C., Aranda F. J., Teruel J. A., Manresa A., Oriz A. and Farfàn M. (2012). Sphingobacterium detrgens sp. Nov., a surfactant-producing bacterium from soil. In *IJSEM Papers in Press*, 3.

- Monteiro S.A., Sassaki G.L., Souza L.M., Meira J.M. and Araujo J.M. *et al*. (2007). Molecular and structural

characterization of the biosurfactant produced by *Pseudomonas aeruginosa* DAUPE 614. In *Chem. Phys. Lipids* 147: 1-13.

- Mora P.L. and Philippot P. (1984). Conservation of Wall Paintings. London: Butterworth & Co.

- Morita T., Konishi M., Fukuoka T., Imura T., Kitamoto D. (2008a). Production of glycolipid biosurfactants, mannosylerythritol lipids, by *Pseudozyma siamensis* CBS 9960 and their interfacial properties. In *Journal of Bioscience and Bioengineering* 105 (5): 493–502.

- Morita T., Fukuoka T., Konishi M., Imura T., Yamamoto S., Kitagawa M., Sogabe A. and Kitamoto D. (2009). Production of a novel glycolipid biosurfactant, mannosylmannitol lipid, by *Pseudozyma parantarctica* and its interfacial properties. In *Applied Microbiology and Biotechnology* 83 (6): 1017-1025.

- Morita T., Konishi M., Fukuoka T., Imura T., Yamamoto S., Kitagawa M., Sogabe A. and Kitamoto D. (2008b). Identification of *Pseudozyma graminicola* CBS 10092 as a Producer of Glycolipid Biosurfactants, Mannosylerythritol Lipids. In *Journal of Oleo Science* 57 (2): 123-131.

- Mukherjee A.K. and Das K. (2005). Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. In *FEMS Microbiology Ecology* 54: 479-489.

- Mukherjee S., Das P. and Sen R. (2006). Towards commercial production of microbial surfactants. In *Trends Biotechnol*. 24: 509-515.

- Mulligan C.N. (2009). Recent advances in the environmental applications of biosurfactants. In *Current Opinion in Colloid & Interface Science* 14: 372–378.

- Myers D. (2006). Surfactant Science and Technology, Wiley.

- Nayak A.S., Vijaykumar M.H. and Karegoudar T.B. (2009). Characterization of biosurfactant produced by *Pseudoxanthomonas sp.* PNK-04 and its application in bioremediation. In *International Biodeterioration & Biodegradation* 63 (1): 73–79.

- Neto D.C., Meira J.A., Tiburtius E., Zamora P.P., Bugay C., Mitchell D.A. and. Dr N.K. (2009). Production of rhamnolipids in solid-state cultivation: Characterization,

downstream processing and application in the cleaning of contaminated soils. In *Biotechnology Journal* 4 (5): 748–755.

- Neu, T.R., Dengler, T., Jahn, B. and Poralla, K. (1992). Structural studies of an emulsion-stabilizing exopolysaccharide produced by an adhesive, hydrophobic Rhodococcus strain. In *Journal of Microbiology* 138: 2531–2537.

- Nitschke M. and Costa S.G. (2007). Biosurfactants in food industry. In *Trends Food Sci. Technol*. 18: 252-259.

- Nordio C. (2001). Restaurare per riscoprire. Restauro del bassorilievo in gesso Endimione Dormiente. Accademia di Belle Arti di Venezia, 15-17.

- Oliveira F.J.S., Vazquez L., de Campos N.P. and de França F.P. (2009). Production of rhamnolipids by a *Pseudomonas alcaligenes* strain. In *Process Biochemistry* 44 (4): 383–389.

- Ortega-Calvo J.J. and Saiz-Jimenez C., (1997). Microbial degradation of phenanthrene in two Europea cathedrals. In *FEMS Microbiology Ecology* 22: 95-101.

- Ortiz A., Teruel J.A., Espuny M.J., Marques A., Manresa A. and Aranda F.J. (2006). Effects of dirhamnolipid on the

structural properties of phosphatidylcholine membranes. In *Int. J. Pharmaceutics* 325: 99-107.

- Palla F. (2006). Le biotecnologie molecolari per la caratterizzazione e la valutazione del ruolo dei microrganismi nei processi di degrado di manufatti di interesse storico artistico. *Quaderni di scienza della conservazione*: 183-194.

- Palla F. and Tartamella E. (2007). Chromatic alteration on marble surfaces analysed by molecular biology tools. In *Conservation Science in Cultural Heritage* 7/2007: 111-127.

- Park A.J., Cha D.K. and Holsen M. (1998). Enhancing solubilisation of sparingly soluble organic compounds by biosurfactants produced by *Nocardia erythropolis*. In *Water Envirnoment Research* 70: 351-355.

- Pei X., Zhan X. and Zhou Z. (2009). Effect of biosurfactant on the sorption of phenantherene onto original and H_2O_2-treated soils. In *J. Environ. Sci.* 21: 1378-1385.

- Peng F., Liu Z., Wang L. and Shao Z. (2007). An oil-degrading bacterium: *Rhodococcus erythropolis* strain 3C-9 and its biosurfactants. In *Journal of Applied Microbiology* 102 (6): 1603–1611.

- Priya T. and Usharani G. (2009). Comparative Study for Biosurfactant Production by Using *Bacillus subtilis* and *Pseudomonas aeruginosa*. In *Botany Research International* 2 (4): 284-287.

- Rahman P.K.S.M., Thahira-Rahman and Lakshmanaperumalsamy P. (2002). Occurrence of crude oil degrading bacteria in gasoline and diesel station soils. In *Journal of Basic Microbiology* 42 (4): 284-291.

- Rai S.K. and Mukherjee A.K. (2010). Statistical optimization of producing, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from *Bacillus subtilis* DM-04. In *Biochem. Eng. J*. 48: 173-180.

- Ranalli G., Alfano G., Belli C., Lustrato G., Colombini M.P., Bonaduce I., Zanardini E., Abbruscato P., Cappitelli F. and Sorlini C. (2005). Biotechnology applied to cultural heritage: biorestoration of frescoes using viable bacterial cells and enzymes. *Journal of applied microbiology* 98: 73-83.

- Ranalli G., Chiavarini M., Guidetti V., Marsala F., Mattini M., Zanardini E. and Sorlini C. (1997). The use of microrganisms for the removal of sulphates on artistic stoneworks. In

International biodeterioration and biodegradation 40: 255-261.

- Riding R. (2000). Microbial carbonates: the geological record of calcified bacterial – algal mats and biofilms. In *Sedimentology* 47:179-214.

- Rinaldi Andrea (2006). Saving a fragile legacy. In *Embo reports* 7(11): 1075-1079.

- Rivadeneyra M.A., Delgado G, Soriano M., , Ramos-Cormenzana A. and Delgado R. (1999). Biomineralization of Carbonates by Marinococcus albus and Marinococcus halophilus Isolated from the Salar de Atacama (Chile). In *Current Microbiology* 39:53-57.

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

- Rodrigues L., Moldes A., Teixeira J. and Oliveira R. (2006a). Kinetic study of fermentative biosurfactant production by *Lactobacillus strains*. In *Biochem. Eng. J*. 28: 109-116.

- Rodrigues, L., H.C.V. Mei, J. Teixeira and R. Oliveira (2004). Influence of biosurfactants from probiotic bacteria on formation of biofilms on voice prostheses. In *Applied Environ. Microbiol.* 70: 4408-4410.

- Rodrigues, L., I.M. Banat, J. Teixeira and R. Oliveira (2006c). Biosurfactants: Potential applications in medicine. In *J. Antimicrobial Chemotherapy* 57: 609-618.

- Rodrigues, L.R., J.A. Teixeira, H.C.V. Meib and R. Oliveira (2006d). Isolation and partial characterization of a biosurfactant produced by *Streptococcus thermophilus* A. In *Coll. Surf B: Biointerfaces* 53: 105-112.

- Rodrigues, L.R., Teixeira J.A. and Oliveira R. (2006b). Low cost fermentative medium for biosurfactant production by probiotic bacteria. In *Biochem. Eng*. J. 32: 135-142.

- Rodriguez-Navarro C., Rodriguez-Gallego M., Ben Chekroun K. and Gonzalez-Muñoz M.T. (2003). Conservation of Ornamental Stone by *Myxococcus xanthus*-Induced Carbonate Biomineralization. In *Applied and Environmental Microbiology* 69 (4): 2182-2193.

- Rölleke S., Muyzer G., Wawer C., Wanner G. and Lubitz W. (1996). Identification of bacteria in a biodegraded wall

painting by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. In *Applied and Environmental Microbiology* 62 (6): 2059-2065.

- Rosen M.J. and Kunjappu J.T. (2012). Surfactantand Interfacial Phenomena (4th ed.). Hoboken, New Jersey: John Wiley & Sons.

- Rosenberg, M. and Rosenberg, E. (1981). Role of adherence in growth of Acinetobacter calcoaceticus RAG-1 on hexadecane. In *Journal of Bacteriology* 148: 51–57.

- Ruggeri C., Franzetti A., Bestetti G., Caredda P., La Colla P., Pintus M., Sergi S. and Tamburini E. (2009). Isolation and characterisation of surface active compound-producing bacteria from hydrocarbon-contaminated environments. In *International Biodeterioration & Biodegradation* 63: 936-942.

- Ruiz-Garc, C., Quesada E., Martnez-Checa E., Llamas I., Urdaci M.C. and Be Jar V. (2005). *Bacillus axarquiensis* sp. nov. and *Bacillus malacitensis* sp. nov., isolated from river-mouth sediments in Southern Spain. In *Int. J. Syst. Evol. Microbiol.* 55: 1279-1285.

- Sabate D.C., Carrillo L. and Audisio M.C. (2009). Inhibition of paenibacillus larvae and ascosphaera apis by *Bacillus*

subtilis isolated from honey bee gut and honey samples. In *Res. Microbiol*. 160: 193-199.

- Saeki H., Sasaki M., Komatsu K., Miura A. and Matsuda H. (2009). Oil spill remediation by using the remediation agent JE1058BS that contains a biosurfactant produced by *Gordonia* sp. strain JE-1058. In *Bioresource Technology* 100: 572-577.

- Saini H.S., Barragán-Huerta B.E., Lebrón-Paler A., Pemberton J.E., Vázquez R.R., Burns A.M., Marron M.T., Seliga C.J., . Gunatilaka A. A. L and Maier R.M. (2008). Efficient Purification of the Biosurfactant Viscosin from *Pseudomonas libanensis* Strain M9-3 and Its Physicochemical and Biological Properties. In *Journal of Natural Products* 71 (6): 1011–1015.

- Saiz-Jimenez C. (1997) Biodeterioration vs Biodegradation: the Role of Microorganisms in the Removal of Pollutants Deposited on Historic Buildings. In *International Biodeterioration & Biodegradation* 40 (2-4): 225-232.

- Santa Annal L.M., Sebastian G.V., Menezes E.P., Alves T.L.M., Santos A.S., Pereira Jr. N. and Freire D.M.G. (2002). Production of biosurfactants from *Pseudomonas aeruginosa* PA1 isolated in oil environments. In *Braz J. Chem. Eng*. 19: 159-166.

- Satirova A.V., Spasova D.I., Galabova D.N., Karpenko E. and Shulga A. (2008). Rhamnolipid-Biosurfactant Permeabilizing Effects on Gram-Positive and Gram-Negative Bacterial Strains. In *Curr. Microbial*. 56: 639-644.

- Schabereiter-Gurtner C., Piñar G., Lubitz W., Rölleke S. (2001). An advanced molecular strategy to identify bacterial communities on art objects. *Journal of Microbiological Methods* 45, 77-87.

- Segal J. and Cooper D. (1977). The use of enzymes to release adhesives. In *The paper conservator*, Vol. 2.

- Simoes M., Simoes L.C. and Vieira M.J. (2010). A review of current and emergent biofilm control strategies. LWT- Food Sci. Technol., 43: 573-583.

- Singh A., Hamme J.D. and Ward O.P. (2007). Surfactants in microbiology and biotechnology. Part 2. Application aspects. Biotechnol. Adv., 25: 99-121.

- Smyth T.J.P., Perfumo A., Marchant R., Banat I.M. (2010) Isolation and analysis of low molecular weight microbial glycolipids. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 3705–3723.

- Sprocati A.R., Alisi C., Segre L., Tasso F., Galletti M. and Cremisini C., (2006). Investigating heavy metal resistance, bioaccumulation and metabolic profile of a metallophile microbial consortium native to an abandoned mine. In *Science of the Total Environment*, Vol. 366: 649-658.

- Sprocati A.R., Alisi C., Tasso F., Vedovato and Barbabietola N. (2007). Esplorazione della tomba della Mercareccia (Tarquinia) per un'analisi descrittiva dei microorganismi colonizzatori e selezione di ceppi di interesse biotecnologico per la conservazione ed il restauro. In : *Atti del Convegno Lo stato dell'arte 5*, IGIIC – gruppo Italiano - the International Institute for Conservation. Cremona (Italy),11-13 Ottobre.

- Sprocati A.R., Alisi C., Tasso F., Vedovato E. and Barbabietola N. (2007). Esplorazione della tomba della Mercareccia (Tarquinia) per un'analisi descrittiva dei microorganismi colonizzatori e selezione di ceppi di interesse biotecnologico per la conservazione ed il restauro. In *Atti del Convegno Lo stato dell'arte 5* - Congresso IGIIC, the International Institute for Conservation. Cremona (Italy), Cremona 11-13 ottobre 2007.

- Sprocati A.R., Alisi C., Tasso F., Vedovato E., Barbabietola N. and Cremisini C., (2008). A Microbiological survey of the Etruscan Mercareccia Tomb (Italy): contribution of

microorganisms to deterioration and restoration. In Art 2008 *Non-destructive investigations and microanalysis for the diagnostics and conservation of cultural and environmental heritage*, Jerusalem, Israel, May 25-30, 2008.

- Sprocati A.R., Alisi C., Tasso F., Vedovato E.,. Barbabietola N and Cremisini C. (2008). A microbiological survey of the Etruscan Mercareccia tomb (Italy): contribution of microorganisms to deterioration and restoration, In *Art 2008- Non-destructive investigations and microanalysis for the diagnostics and conservation of cultural and environmental heritage*, Jerusalem, Israel.

- Steyn P.L., Segers P., Vancanneyt M., Sandra P., Kersters K. and Joubert J. J. (1998). Classification of heparinolytic bacteria into a new genus, *Pedobacter*, comprising four species: *Pedobacter heparinus*comb. nov., *Pedobacter piscium* comb. nov., *Pedobacter africanus* sp. nov. and *Pedobacter saltans* sp. nov. Proposal of the family *Sphingobacteriaceae* fam. Nov. In IJSEM, January 1998, vol. 48, 1: 165-177.

- Stiller J.W. and McClanahan A. (2005). Phyto-specific 16S rDNA PCR primers for recovering algal and plant sequences from mixed samples. In *Molecular Ecology Notes* No.5: 1-3.

- Stulik D., Khanjian H., Dorge V. and de Tagle A. (2002). Scientific investigation of surface cleaning processes: quantitative study of gel residue on porous and topographically complex surface. Scientific methods of examination of works of art. ICOM Committee for Conservation.

- Tasso F., Alisi C., Salluzzo, Tammaro M., Ubaldi C., Cremisini C. and Sprocati A.R. (2008). Treatment of tannery effluents in BIO and BAC bioreactors inoculated with the native microbial consortium ENEA-CAR. In *14th International Biodeterioration and Biodegradation Symposium*, Messina 6-11 October.

- Techaoei S., Leelapornpisid P., Santiarwarn D. and Lumyong. S. (2007). Preliminary screening of Biosurfactant producing Microorganisms isolated from hot Spring and garages in northern Thailand. Kmitl sci. Tech. J. Vol. 7 no. S1 38-43.

- Thavasi R., Subramanyam Nambaru V. R. M., Jayalakshmi S., Balasubramanian T., Banat I.M. (2008). Biosurfactant Production by *Azotobacter chroococcum* Isolated from the Marine Environment. In *Marine Biotechnology* 11 (5): 551-556.

- Tiano P. (1998). Biodegradation on cultural heritage: decay mechanisms and control methods. In *Science and Technology for Cultural Heritage* 7: 19-38.

- Tiano P. and Cantisani E. (2005). Precipitazione bioindotta di calcite per la conservazione delle pietre monumentali, *Progetto Bioreinforce* – (EVK4 –2000-00037).

- Tiano P., Biagiotti, L. and Mastromei G. (1999). Bacterial bio-mediated calcite precipitation for monumental stones conservation: methods of evaluation. In *Journal of microbiological methods* 36: 139-145.

- Toledo F.L., Calvo C., Rodelas B. and Gonzàlez-Lopez J. (2006). Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. In *Systematic and Applied Microbiology* 29: 244-252.

- Tuleva B., Christova N., Cohen R., Antonova D., Todorov T. and Stoineva I. (2009). Isolation and characterization of trehalose tetraester biosurfactants from a soil strain *Micrococcus luteus* BN56. In *Process Biochemistry* 44 (2): 135–141.

- Tuleva B., Christova N., Cohen R., Stoev G. and Stoineva I. (2007). Production and structural elucidation of trehalose tetraesters (biosurfactants) from a novel alkanothrophic *Rhococcus wratislaviensis* strain. In *Journal of Applied Microbiology* 104: 1703-1710.

- Urzì C., Garcia-Valles M., Vendrell M. and Pernice A. (1999). Biomineralization processes on rock and monument surfaces observed in field and in laboratory conditions. In *Geomicrobiological journal*, 16: 39-54.

- Van Hamme, J.D., Singh, A., Ward, O.P. (2006). Physiological aspects. Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. In *Biotechnology Advances* 24: 604–620.

- Vollenbroich D., Ozel M., Vater J., Kamp R.M., Pauli G. (1997). Mechanism of Inactivation of Enveloped Viruses by the Biosurfactant Surfactin from *Bacillus subtilis*. In *Biologicals* 25: 289-297.

- Wang S. and Mulligan C.N. (2009). Rhamnolipid biosurfactant-enhanced soil flushing for the removal of arsenic and heavy metals from mine trailing. In *Proc. Biochem*. 44: 296-301.

- Webster A. and May E. (2006). Bioremediation of weathered-building stone surfaces, *TRENDS in Biotechnology* Vol.24, No.6.

- Wendelbo O. (1970). The use of proteolytic enzymes in the restoration of paper and papyrus. University library of Bergen.

- Wheeler .P. and Sikes C.S. (1989). Matrix-crystal interaction in $CaCO_3$ biomineralization. In *Biomineralization Chemical and Biochemical Perspectives*, pp. 95-132. VCH. New York.

- Wolbers R. (1990). Notes for workshop on new methods in the cleaning of paintings. Getty conservation institute, Marina del Rey.

- Youssef N.H., Duncan K.E., Nagle D.P., Savage K.N., Knapp R.M. and McInerney M.J. (2004). Comparison of methods to detect biosurfactant production by diverse microorganisms. In *Journal of Microbiological Methods* 56: 339-347.

- Zhang Y. and Miller R.M. (1992). Enhanced octadecane dispersion and biodegradation by a Pseudomonas rhamnolipid surfactant (biosurfactant). In *Applied and Environmental Microbiology* 58: 3276–3282.

- Zhang Y. and Miller R.M. (1995). Effect of rhamnolipid (biosurfactant) structure on solubilisation and biodegradation

of n-Alkanes. In *Applied and Environmental Microbiology* 61 (6): 2247–2251.

- Zouboulis A.I., Matis K.A., Lazaridis N.K. and Golyshin P.N. (2003). The use of biosurfactants in flotation: Application for the removal of metal ions. In *Mineral Eng*., 16: 1231-1236.

WEBSITES

- http://ec.europa.eu/research/environment/pdf/survey_and_outcomes_cultural_heritage.pdf
- http://www.scientificamerican.com/article.cfm?id=the-science-of-saving-art
- http://www.heritageportal.eu/index.php?option=com_content&view=article&id=1170%3Anew-european-projects-on-cultural-heritage-research&catid=1%3Alatest-news&Itemid=637&lang=en
- http://www.scialert.net/fulltext/?doi=ajdd.2011.58.69&org=11
- http://www.wmf.org/project/casina-farnese-palatine-hill

CITED THESIS

- Barbabietola N. (Anno Accademico 2005/2006) Isolamento di ceppi batterici calcinogenici da un ipogeo etrusco ed ipotesi di una loro applicazione nel biorestauro. Università degli Studi di Roma La Sapienza.
- Barbabietola N. (PhD Thesis in Science for Conservation of Cultural Heritage – XXIV Cycle) Innovative microbe-based technology for Conservation and restoration. University of Florence.
- Botticelli M. (Anno Accademico 2006/2007) Screening Di Microrganismi Per La Rimozione Di Gommalacca, Applicata Come Fissativo Su Dipinti Murali. Università degli Studi di Roma La Sapienza.
- Fiorillo F. (Anno Accademico 2006/2007) Screening Di Microrganismi Per La Rimozione Di Cera E Resina, Usate In Miscela Nella Tecnica Della Foderatura. Università degli Studi di Roma La Sapienza.
- Grimaldi M. (Anno Accademico 2007/2008) Studio di microrganismi per lo sviluppo di tecniche di biorestauro. Università degli Studi di Roma La Sapienza.