BIOTEHNOLOGII ECOLOGICE FOLOSITE ÎN RESTAURAREA PICTURII MURALE ȘI A SUPORTULUI LITIC: REVIEW

GREEN BIOTECHNOLOGIES USED IN THE RESTORATION OF MURAL PAINTING AND LITHIC SUPPORT: REVIEW

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The development of effective and non-destructive conservation strategies is based on understanding the role of microorganisms as biodeteriogens and their metabolites in biocleaning and biological control. An efficient alternative to traditional strategy in restoration is biorestoration based on different green biotechnologies where microorganisms and their enzymes are main actors. They are used both in bioconsolidation due to their ability to produce calcium carbonate precipitation and biocleaning. Microorganisms are used as viable cells brushed on the art works or embedded in carriers. Biorestoration is characterised by low cost and invasiveness, high specificity, easy control, non-toxicity for restorers and friendly for environment. Due to the fact that biorestoration has advantages and disadvantages, a better information of conservators and restorers in the use of the biotechnologies applied to restoration and conservation together with new results on optimizing the biocleaning and bioconsolidation about microorganisms, enzymes and bioactive molecules is needed. They will contribute to technological transfer on a large scale.

Dezvoltarea strategiilor eficiente și nedistructive de conservare se bazează pe înțelegerea rolului microorganismelor ca biodeteriogeni, în producerea metaboliților lor cu rol în biocurățare dar și în activittaea de control biologic. O alternativă eficientă la strategia tradițională de restaurare este biorestaurarea bazată pe diferite biotehnologii ecologice în care microorganismele și enzimele produse de acestea au rolul decisiv. Acestea se folosesc atât în bioconsolidare datorită capacității de a produce precipitarea carbonatului de calciu cât și a biocurățării. Microorganismele se folosesc atât ca celule viabile care se aplică prin pensulare pe suprafața operei de artă cît și incluse în diferite suporturi. Procesul de biorestaurare este caracterizat prin costuri și invazivitate scăzute, înaltă specificitate, control facil, absența toxicității față de restauratori și mediu. Datorită faptului că biorestaurarea are avantaje și dezavantaje este necesară o mai bună informare a conservatorilor și restauratorilor referitoare la folosirea biotehnologiilor în restaurare și conservare, rezultatele recente asupra optimizării biocurățării și bioconsolidării și la microorganismele producătoare de enzime și molecule bioactive. Acestea vor contribui la transferul tehnologic la scară largă.

Keywords: *microorganisms, biotechnologies, biconsolidation, biocleaning, biological control, mural paintings, stone*

1.Introduction

Microorganisms have a significant impact on cultural heritage but at the same time are very effective in its biotreatment. Green biotechnologies used in the restoration are an alternative to conventional treatments being friendly with the artwork, the environment and the restorer.

When an artwork is damaged restoration strategy is developed. In this respect restorers get information about microorganisms involved in biodeterioration and act to stop or minimize their metabolic activity in accordance with the state of preservation of the artwork. Microbial compounds such as calcium carbonate is used for biotreatment of stone, calcium oxalate films are protective layers and enzymes become cleaners.

The paper is divided into three thematic sections. The first describes the negative effect of microorganisms on conservation because they damage the artwork. The second analyzes microorganisms as potential source used in biotechnological treatments of historical monuments. The third presents the main green biotechnologies in the conservation of mural painting and lithic support: bioconsolidation, biocleaning and biological control.

2. Microorganisms as biodeteriogens of historical monuments

Archaea, bacteria, fungi and lichens grow on historical monuments having a significant potential to deteriorate them (Fig.1). There are guidelines to preserve indoor mural paintings mostly for humidity and temperature control which are very important for their growth. Outdoor murals and stone are much more vulnerable to damage due to changing

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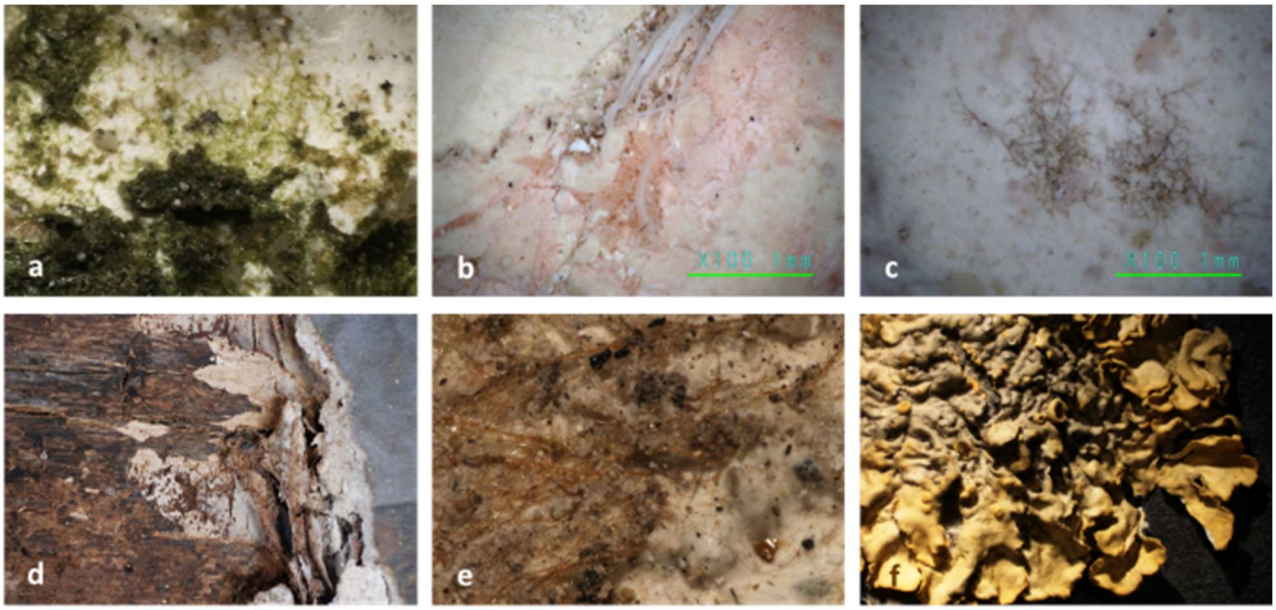

Fig. 1 - Different biodeteriogens colonizing mural paintings (a-green alga; b-pink bacteria; c-melanic fungi; d- melanic fungi and basidiomicetes, e-basidiomycetes) and stone (f-lichens) / *Diferiți biodeteriogeni colonizatori ai picturilor murale (a-alge verzi; b-bacterii cu pigmentație roz; c-fungi melanici; d-fungi melanici și bazidiomicete; e-bazidiomicete) și ai pietrei (f-licheni).*

environmental parameters and very poor prevention assessment [1]. Using different methods there have been isolated bacteria such as *Acinetobacteria* sp., *Bacteroides* sp., *Flavobacterium* sp., *Micrococcus* sp., *Sarcina* sp., [2], *Paenibacillus* sp., *Pseudomonas* sp. [3], *Thiobacillus* sp [4] and fungi such as *Alternaria* sp, *Aspergillus* sp. [5], *Coniosporium* sp,. *Exophiala* sp., *Hortea* sp., *Knufia* sp. [6] and *Trichoderma* sp. [7]. Some bacteria and green algae (*Gloeocapsa*, *Phormidium*, Chorococcus, *Chlorella*, *Stichococcus*, and *Chlorococcum*) [4] are involved in the development of biofilm with potential production of mechanical stress to mineral matrix leading to changes such as pore size, water absorbion and temperature response.

Streptomyces spp. [8], *Aspergillus* sp. and *Penicillium* sp. are involved in pigment and enzymes production [9]. Microorganisms such as *Gloeocapsa* sp., *Rubrobacter* sp., *Aspergillus* sp. and *Penicillium* sp., colonize both canvas paintings and murals [3, 5, 10, 11]. Lichens are primary colonizers in the biodeterioration of stone [12].

The main mechanisms of biodeterioration are: *discoloration* as a consequence of pigmented cells and/or cellular pigments, *encrustation and exfoliation* after fungal hyphae penetration into substrate, *complexation and release of cations* produced by exopolysaccharides or organic acids *or secondary mineralization and crystallization* as a result of the deposition of gypsum [13].

The research is focused to take into account more ecological considerations for establishing the sustainable conservation strategies. In this respect, the identification of biodeteriogens and their mechanisms of deterioration have to be followed by rate assessment before taking preventive measures based on the on-site biodeterioration assessment [13]. Presently there are specific techniques able to provide information on the microbial communities which grow on the surface of artworks, their biodeterioration potential, their control and prevention of a new growth.

3. Microorganisms used in biotechnological treatments of historical monuments

The ability of non-pathogenic microorganisms to use mineral or organic deposits, as nutrients is important for biocleaning and their potential is applied by different biotechnologies in cultural heritage. Research is focused on isolation of microorganisms from natural environments and from the biodeteriorared artwork, testing them for ability to produce metabolites of interest (enzymes, acids, antibiotics) optimizing biosynthetic conditions and developing biotechnology (Fig.2). The short bio-application contact time of advanced agar-gauze gel activated with viable *P. stutzeri* cells is an alternative method to the traditional *on-site* cleaning techniques currently in use for altered historical wall paintings [14, 15]. Treatment of artworks with metabolites (mostly enzymes) in cultural heritage restoration is currently limited because their efficiency depends on environmental stability [1]. Gherardi et all. [16] obtained very good results in case of cleaning of heritage textiles with enzymes immobilized on gold nanoparticles. They improved the efficiency of enzymatic treatment by increasing its resistance to environmental conditions up to 5-fold. This technique could be also applied for murals.

Innovative strategies for sustainable conservation of historical monuments are based on *in situ* bio-intervention mainly bioconsolidation, biocleaning and biological control. Exploration of

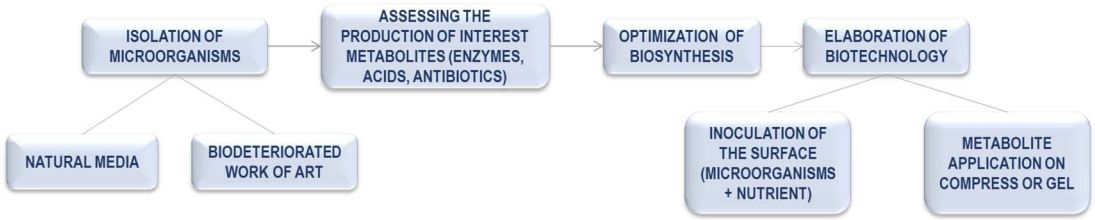

Fig. 2 - Development of a biotechnology in the conservation field based on the use of microorganisms / *Dezvoltarea unei biotehnologii în domeniul conservării bazată pe folosirea microorganismelor*

novel functional nanomaterials such as TiO_2, ZnO and Ag with photocatalytic, antifouling and antibacterial properties open a new window in sustainable conservation of artworks [13].

4.Green biotechnologies in the conservation of mural painting and lithic support

Biotehnologies are used in the field of cultural heritage conservation as consequence of the positive effects of microbial metabolites of non-pathogenic microorganisms (bacteria and fungi). Microbial biotechnologies are alternatives to traditional methods because they are effective, non-aggressive and non-potential dangerous for object, restorers and environment. Research is focused mainly on biorestoration which includes bioconsolidation, biocleaning and biological control.

4.1. Bioconsolidation

Biological mineralization is an important process for bioconsolidation and protection of stone monuments and historic buildings, elaboration of new building materials, soil stabilization, carbon dioxide capture and drug delivery [17].

4.1.1. Bioconsolidation and protection of stone monuments and historic buildings

Bioconsolidation, based on ability of bacteria to precipitate $CaCO_3$ has been proposed for restoration of monuments and historic heritage buildings.

In microbial induced calcite precipitates by urea hydrolysis, the enzyme urease catalyses substrate urea and precipitates carbonate ions in presence of ammonium. These carbonate ions in the area with a calcium source readily precipitate calcium carbonate. The urea hydrolysis is the result of metabolic processes, which depends on the type of bacteria used. Increased microbial induced calcite production was observed by using co-culture of ureolytic and non ureolytic bacteria (*Sporosarcina pasteurii* and *Bacillus subtilis*).

Bioprecipitation of calcium carbonate is also performed by bacteria able to develop biofilm. The nature and composition of biofilms have an impact on morphology, bioaccumulation and $CaCO_3$ formation mechanisms and crystal geometry [17]. *Bacillus licheniformis* and *Lysinibacillus sphaericus* produce $CaCO_3$ with different morphologies [18]. *Pseudomonas putida* INQCS 113, *Lysinibacillus sphaericus* INQCS 414 and *Bacillus subtilis* INQCS 328 produce $CaCO_3$ with different polymorphisms [19].

Calcium carbonate bioprecipitation takes place by biological control where bacteria control the process independently of environmental conditions and by induced mineralization where type of mineral produced by bacteria is almost totally dependent on environmental conditions. Calcinogenic bacteria have several genes involved in crystal formation. The process is controlled by the following factors: temperature, pH, concentration of calcium ion, concentration of carbonate ion, concentration and diffusion rates of nutrients and metabolites as well as the availability of nucleation places. Bacteria release two minerals (calcite and vaterite) which are accumulated in the porous spaces of the lithic objects, restoring their cohesion.

Bioconsolidation is an eco-friendly technology based on biocarbonatogenesis produced by calcinogenic bacteria. In fact, it is a natural process that occurs in many environments and is related to the formation of carbonate sediments in rocks [20]. Design experiments and biotechnologies require information about biological processes (growth, metabolism, specific enzymatic activities) chemical reaction responsible for formation of insoluble compounds, precipitation, crystallization and adhesion.

In passive carbonatogenesis, bacterial activity produces chemical changes in microenvironment, leading to the accumulation of carbonate and bicarbonate ions and to the precipitation of solid particles [21]. In this process are involved metabolic pathways of the sulphur and nitrogen cycle.

In active carbonatogenesis the carbonate particles are produced by ionic exchanges through the cell membrane [21].

Bacterial cell wall contains functional groups (phosphates, hydroxyls, carboxyls) very reactive to metallic ions such as Ca^{2+}. They become nucleation centres on which the subsequent minerals are formed incorporating bacterial cells.

Very efficient in calcite precipitation for conservation purpose are the following bacteria: *Bacillus pasteurii* [22], *Bacillus subtilis* [23], *Myxococcus xanthus* and *Pseudomonas putida* [24].

Three hypotheses have been proposed for biological mechanisms of calcium carbonate precipitation [25]. According to the first hypothesis

mineralization occurs as by-product of microbial metabolism involving either autotrophic or heterotrophic pathways. Heterotrophic bacteria from nitrogen and sulphur cycle by enzymatic hydrolysis of urea or disassimilatory reduction of nitrate and sulphate increase pH, shifting the carbonate-bicarbonate equilibrium towards the production of more carbonate ions. Calcium ions being free, precipitation of calcium carbonate (passive carbonatogenesis) takes place. The second hypothesis suggests that carbonate nucleation takes place on the cell wall. By successive stratification, bacteria are embedded in growing carbonate crystal. The third hypothesis attributes the essential role to proteins present in extracellular polymeric materials (active carbonatogenesis).

Bioconsolidation of carbonate stone monuments depends on bacterial species, environmental conditions (temperature, relative humidity and solution ion content), the proper nutrients and reaction kinetics. Bacteria after a latency period grow rapidly and become nucleation centres. Generally all bacteria isolated from historical monuments are able to induce carbonate precipitation under laboratory conditions.

One of the bioconsolidation biotechnologies is starting with the growth of bacteria in culture media and then is sprayed onto deteriorated limestone. They are usually fed with nutrient spray daily or every two days. As result, a superficial calcareous layer thick of some microns is produced. This technology has been improved by effort to find more effective bacteria in order to provide more calcite, less costly nutrient and better match between the types of limestone, the nurturing sequence and the type of bacteria [24]. The application of living cultures of selected biocalcifying bacterial strains can produce a superficial coating, called biocalcin. The advantage of this treatment is to obtain a mineral product very similar to that of the stone substrate, mimicking the natural process responsible for calcareous stone formation.

The other biotechnology for bioconsolidation is starting with nutrient spray to activate calcinogenic bacteria from the microbial community of stone or airborne contaminants as eco-friendly methods for *in situ* consolidation of stone monuments. Panella [20] developed a biotechnology based on the use of culture medium called M-3P (Bacto Casitone) as the source of carbon and nitrogen for calcinogenic bacteria. Bioconsolidation was compared with the conventional consolidants frequently used to restore the cohesion of painted and stucco plasters: colloidal nanoparticles of $Ca(OH)_2$ and acrylic resin. Experiments have been performed both on experimental models and detached erratic fragments of painted plaster and Roman stucco. All samples covered with the already mentioned products had been placed in Chapel of St. Peter in the church of Santa Pudenziana in Rome, Italy. Evaluation of effectiveness performed by contact sponge methods, peeling tape test, contact angle measurement on samples of painted plaster and Roman stucco, SEM analysis on detached erratic fragments revealed positive results. Visual analysis, colorimetric measurements and biological growth control support the application of this biotechnology because it does not require the use of chemicals and solvents, it is easy to apply and it is compatible with carbonate substrates.

Bioconsolidation can be also be based on applying a biotechnology which uses bacterial metabolites (proteins) or extracellular polymeric materials for calcium carbonate precipitating.

The bacteria induce calcium carbonate, reduce water permeability of the treated stone, increase its durability and improve mechanical properties of the material [26]. Reduction in water absorption can be 50% in case of limestone samples [24]. De Muynck et al. [27] found that the pore structure has a great impact on the effectiveness of a biogenic carbonate surface treatment for limestone conservation. Bacterial generated carbonates were more successful in macroporous stone (higher extent, at greater depths).

Pérez et al. [17] characterized the formation of crystals by *Bacillus subtilis* in a semi-solid non-ureolytic system, focusing on the potential applicability to producing biomaterials and the development of a novel protective system with self-healing ability. In this respect, scientists inoculated *Bacillus subtilis* ATCC 23857 strain onto nutrient agar supplemented with 0,025 M calcium acetate. After incubation at 37^0C for 3 days, crystals exhibited a size varying from 50 to 170 μm and rounded aspects with a defined border and pyramidal centre. The crystal growth pattern showed central and radial expansion in accordance with colonial morphology. During the growth, bacteria produced capsule containing exopolymeric compounds which interact with dissolved metals. Surfactin interact also with calcium ions reducing the surface tension and decreasing colony size. Due to metabolic processes, the increase of pH in the surrounding environment takes places. It is considered that capsule and surfactin molecules promote crystal precipitation. The elemental composition of bacterial crystals evaluated by energy-dispersive X-ray spectroscopy confirmed that they are $CaCO_3$. Calcite was the metastable and predominant phase (82%) and vaterite was unstable and small one (13,8%).

The stability of amorphous material may be related to the interaction of precipitates with exopolymeric compounds allowing metastability towards environmental conditions [28].

4.1.2 Development of novel cement-based materials

The application of biomineralization process is important for building industry. Intensive research is developed for repair systems of concrete and

reinforced concrete failure, cement binders, porous fillers, coating systems and self-healing systems in cementations materials [17].

Wiktor and Jonkers [29] implemented on pilot scale a new material decreasing water permeability by 47% after 2 months by application. The main risk factor is sensitivity of bacteria under environmental and physicochemical condition of concrete.

Wu et al. [30] used the biocimentation ability of *Bacillus* species for biogrouting of rocks. They developed a new control method for fractured rock based on biogrouting produced by microbial induced calcite precipitation process. They also performed a study on the spatial distribution of biogrout in a rock fracture and its effect on permeability reduction. The most important factor to achieve calcite precipitation throughout the mass is uniform distribution of microbial cells followed by fixation inside the porous structure. Several ways of introducing carbonatogenic bacterial cells have been developed. Prior mixing of bacterial cells and cement material leads to immediate flocculation of bacteria and crystal growth which may play an important role in treatment of surfaces. This could lead to rapid clogging of injection point and surrounding areas pore space in case of the fine or medium sand. The two-phase injection is another strategy where the bacterial cell suspension is injected first, followed by the cementation solution. This strategy prevents crystal accumulation around the injection point and leads to a more homogeneous distribution of calcium carbonate. A more uniform distribution of calcite precipitation was achieved over a greater distance in the sand [31].

A cement-compacted concrete, self-compacted was produced using *Bacillus* species and the same ingredients of normal concrete, but using waste materials from industries and agricultural products. The new material has improved construction quality, faster construction activity, reduced cost and others.

Biological mortar had been developed by Université Pierre et Marie Curie, France for the restoration of small voids on limestone surfaces [24] avoiding some difficulties such as chemical and physical incompatibilities (thermal expansion, hardness, porosity) of normally used repair mortars with the original stone material [24]. Biological mortar was evaluated by visual appearance, cohesion properties and occurrence of micro-cracks (performed both during and after drying). The composition of biological mortar is optimized by evaluation of quantity and composition of fine stone powder, nutrients, concentration of inoculum and matching of colour with the original stone. The best results have been obtained by inoculation of 109 cells/ml^{-1}, a fixed amount of nutrient medium and a double amount of fine limestone powder [24].

An attempt was made to enhance the properties of ordinary Portland cement (OPC) using soil microbial solution with lentil seed (*Lens culinaris*) powder as protein source and sugar as carbon source. Bio-OPC specimens using microbial solution instead of water were prepared. Significant increase in compressive strength was observed in bio-OPC specimens as compared to the specimens prepared using water [32]. It was observed that, there was up to 23.49% enhancement in the compressive strength of bio-OPC in 28 days. It was found that the calcium carbonate gets deposited in the pores of matrix of the bio-OPC. Water absorption capacity of bio-OPC was reduced up to 15.40% by filling the micro-cracks in the bio-OPC by the technique of biocementation. Use of *Lentil* seed powder as the source of protein and sugar as the source of carbon in nutrient medium became highly economical.

Presently, bio-based fibers including vegetal fibers of bamboo and hemp are used to reinforce cement-based composites, providing advantages of being more environmental friendly than synthetic fibers, and enhancing the toughness of cementitious mortars. Fungi having mycelial structures with biomass higher than bacteria could also serve as fiber in improving durability or strength of cementitious materials [33]. Fungal mycelia of *P. chrysogenum* CS1 (urease-positive) was used as fiber in cementing sand in a column to form bio-sandstone leading to enhancement of compressive strength. The formed bio-based fiber was visualized under scanning electron microscopy (SEM), the biocementation process was confirmed by Fourier transform infrared spectroscopy (FTIR), and calcite was identified by X-ray diffraction (XRD).

4.1.3.Advantages and disadvantages of bioconsolidation

Biotechnologies applied for bioconsolidation have positive and negative effects [24,31].

The following advantages have been identified:

- The layer of calcite is compatible with historic carbonate stone buildings and sculptures.
- Improvement of mineral precipitation in micro pores at nanoscale range.
- Improvement of coherence and cementation of new formed calcite structure with a consolidation.
- The process can be improved by identification of new bacteria and fungi strains.
- New calcite layer, which is regular and homogeneous, acts as surface protectant of monuments exposed to erosion and pollution.
- It is an ecological alternative to traditional consolidation treatments.
- In macroporous stone is efficient due to calcite growth.
- Retention of the permeability was evident by the absorbance of water recorded in the biocemented surfaces. For consolidation of loose material, it is important to conserve the permeability

so that the water moves through the voids in the stone hindering the deterioration.

- Microbially induced precipitation process is cost effective too as bacterial cells used can be reused several times (from 2-3 times to 20 times). Bacterial enzyme could be reused in subsequent applications of calcium and urease only.
- Bacteria are tolerant towards extreme conditions of cement which makes them better source material for biocementation process.

Even the advantages are obvious, certain reservations and/or limitations regarding the use of biotechnologies based on bioprecipitation of calcium carbonate are debated. In the field of restoration it is not yet possible for biorestoration to be widely applied:

- Bioconsolidation is not efficient for stones with lower porosity and smaller pores.
- The surface on which the biorestoration is applied has to be cleaned before.
- Bioconsolidation showed poor depth of penetration.
- New calcite is thin but dense and crust decreases absorption properies of stone.
- The treated surface has to be evaluated regularly for biological growth because culture media can induce microbial colonization.
- Possible plugging of pores.
- Commercial use of ureolytic bacterial strains may cause problems because urease enzyme is associated with increased virulence among pathogenic bacterial strains.
- Unknown bacterial species under the subsurface are a difficult task that is improved due to complexity of indigenous microbial consortia and their ecological function.Reduction of precipitation occurs in presence of ammonia and high concentration of ammonia may possess threat to human and animal health.
- Gene transfer and mutation may cause unwanted changes in metabolism.

4.2.Biocleaning

Innovative biocleaning method of artworks is based on versatile microbial metabolism. Some microbial enzymes are able to remove complex deposits which are sometimes impossible to remove by conventional methods. In addition, some of the conventional cleaning methods are aggressive and invasive towards the artwork and dangerous for restorers' health.

Presently, the removal of different substrates (animal glue, transparent dispersion of casein, waxes, animal and vegetable fats, acrylic resins) is performed with viable microbial cells or purified enzymes.

Microbial strains are provided from culture collections or are isolated directly from natural environment and the contaminated surface. Cultural-dependent (isolation) and cultural independent (multi-omics= Next Generation sequencing on DNA and RNA, volatile compounds) techniques are used to select the proper strain for specific metabolic activities and safety [34].

The success of the biocleaning process depends on: microorganisms, water, viscosity, delivery system, safety, costs and previously used organic compounds.

The main groups of microorganisms used in biocleaning treatments are: sulfat reducing bacteria, nitrate reducing bacteria (denitrifiers), organic matter degrading bacteria, archaea (extremophiles), heterotrophic bacteria and fungi.

Water free or entrapped in water-based gel is very important both to maintain alive and active the microorganisms and to assist enzymatic chemical reaction. Due to the fact that water becomes a risk for porous and fragile material or when superficial alterations are present it is recommended to control water addition and its use in formulates [34].

Viscosity is another important aspect of water properties. The best densifier agents are: sepiolite, cellulose chemical pulp, cellulose ethers (Klugel, tylose MH) polyacrylic acid derivatives (Carbogel, Carbopol). Agar gel had been proposed by Bosh-Roig [35] because allows a controlled supply of water, a good contact and adhesion with the surface in vertical or inclined position and does not release the residues on the cleaned area.

The main delivery systems for biocleaning are: the sepiolite, the hydrobiogel-97, the cotton wool, Carbogel, mortar and alginate beads, agar, laponite and arbogel [34].

Safety must be ensured for restorers and for the work of art. Biocleaning is based on the use of non-pathogenic microorganisms. The biocleaned surface control is required to assure that there are no microorganisms left after treatment.

The evaluation of the final cost of biocleaning takes into account both negative and positive effects. The effect on human health and environmental protection is highly important (in terms of source, manufacturing process and final residues).

Previous treatments with organic compounds (animal and vegetal glues, varnishes, temperas etc) became in time nutrients for the microorganisms. They are also interacting with the surface of artwork inducing accumulation, developing of deposits and incrustations.

Based on our expertise and summarizing various studies, we consider that the development of a cleaning as a biotechnology requires the following steps:

- Screening of microbial strains from new isolated or from culture collection.
- Evaluation of their efficiency in order to select the best ones.
- Establishing the proper delivery system to carry microbial viable cells or enzymes.
- Evaluation of the biomass and the optimal conditions for microbial metabolism.

- Biocleaning tests on experimental models and on real altered fragments.
- Ensuring the optimal environmental parameters in accordance with those of the species biology and with the conditions for carrying out the enzymatic reactions.
- Removal of the cells and chemicals.
- Short-medium and long-term monitoring of the biocleaned surface.
- Cost evaluation

4.2.1. Biocleaning case studies with viable microbial cells

Biotreatment for removal of nitrates and sulphates by embedded *Pseudomonas pseudoalcaligenes* KF707 and *Desulfovibrio vulgaris* ATCC 29579 in Carbogel, showed after six years, low concentrations of salts, no microorganisms and no colour variation of the stone of Matera Cathedral (Italy) [35]. Arbocel with *Desulfovibrio vulgaris* subsp. *vulgaris* combined with chemical treatment removed the grey deposits and black crusts from the marble columns and statues in Monumental Cemetery, Milan, Italy.

On the external walls of Florence Cathedral, three different cleaning treatments had been applied: chemical poultice (10% ammonium carbonate), laser (Nd: YAG 1064 nm), biocleaning with *Desulfovibrio vulgaris* subsp. *vulgaris* ATCC 29579 embedded in Carbogel. Optical and SEM-EDS microscopy, FTIR spectroscopy and colorimetric measurements revealed that the microbial cleaning was the best method for the removal of gypsum.

Laponite and *Cellulosimicrobium cellulans* removed calcium sulphate and calcium carbonate from Casina Farnese wall painting (Palatine Hil, Rome, Italy) and *D. vulgaris* in hydrobiogel-97 removed black crusts from altered stone surfaces in Failaka Island, Kuwait [36].

Bosch-Roig and Ranalli [37] used *P. stutzeri* DSMZ 5190 strain immobilized into agar gel for 90 min contact time to clean nitrates. The monitoring of the biocleaning treatment showed a 92% reduction of nitrates and no viable cells.

In order to recover the Camposanto Monumental frescoes, the following steps were taken: physical-chemical analysis of the residual adhesive organic matter used in the past to detach the fresco; development and improvement of an advanced biocleaning system to remove this organic matter from the fresco surface; short- and medium-term microbial monitoring; cost-analysis of the biotechnological system employed and the bacterial cells used [38]. As result, it was concluded that a a single 3 hours biocleaning treatment is efficient to remove animal glue.

On San Nicolas church frescoes, Valencia, Spain organic matter residues were removed with *P. stutzeri* DSMZ 5190 in agar gel after 4 hours [34].

Manzzoni et al. [39] cleaned deposits of gypsum, weddellite, calcium carbonate, apatite, nitrate and aged proteinaceous material from the wall paintings of loggia of the Casina Farnese (Palatine Hill, Rome, Italy) with *Cellulosimicrobium cellulans* TBF11E (to clean black crust), *Stenotrophomonas maltophila* UI3E (to remove protein deposits) and *P. koreensis* UT30E (to clean both mixture of inorganic and protein deposits). Duration of applications was 24-48 hours.

At Vatican Museum on the Cristo che salva Pietro dalle acque – La Navicella by G. Lanfranco and on the oil mural painting L'Incarnato by O. and G. Riminaldi inside the Cupola at Pisa Cathedral, Ranalli at al. [14] applied an advanced biocleaning system based on agar-gauze activated gel. The high effectiveness of the treatment was confirmed by Py/GC-MS and FTIR analysis.

Hi-tech restoration by two steps biocleaning process of fresco Triumph of Death at the Camposanto Monumental Cemetry (Pisa, Italy) was performed by Ranalli et al [40]. Initially, the fresco was detached from the walls and glued in a framework screwed onto asbestos cement supports and for biocleaning it was again detached from the support. The animal glue and residues of calcium caseinate were removed from the front and the back in 3 hours after treatment with *P. stutzeri* A 29. The main cleaning steps were: soft cleaning to remove dirt and dust using Japanese paper and water, applying bacterial cell suspension to the surface of fresco covered by paper and hydrophilic layer, soft cleaning by distilled water (using a sponge), chemical cleaning (using an anion exchange resin) to remove the organic residues.

Elhagrassy [15] performed a similar experiment in laboratory and *in situ* applying *Pseudomonas stutzeri* cells on the aged replica. Experiment has been performed in a controlled room chamber using two bacterial cells carriers (cotton and agar) for different periods (60, 120 and 180 min) at 35^0C. At the end of each treatment the carriers were removed and the treated areas were washed with a sponge impregnated with sterile distilled water. *In situ* mural paintings in Ali kadkhoda house (Cairo, Egypt) the following steps were performed: mechanical cleaning with brushes and wet cotton, applying of medical gauze and agar with *P. stutzeri* and broth animal glue on the mural painting for 3 hours, coating by poly ethylene to save the flexibility of agar, removing the poly ethylen, gel agar and medical gauze, applying nano silver particles and pre-consolidation by Paraloid B-72. XRD, FTIR, SEM-EDX and microbial analysis showed the efficiency of the biorestoration.

Romano et al. [41] found that *Halomonas campaniensis* was very efficient for removal of nitrate crust of stonework due to the ability of this bacterial species to adapt to very low or high temperatures, extreme values of pH, high salt

concentration, high pressure, heavy metals and radiation.

4.2.2. Biocleaning case studies with enzymes

Biocleaning with enzymes is an alternative to chemical treatment with a lower risk for artworks, human health and environment. It is also more selective and less aggressive for artworks. Enzymes are provided by plants, animals and microorganisms. The main enzymes used for artworks biocleaning are: esterase-Lipase, amylase, cellulase and protease. The most efficient are esterase-lipase produced by *Candida cilindracea* (removal of oil residues, resins, wax or Paraloid B-72), α-amylase produced by *Bacillus* sp. and *Aspergillus* sp. (detachment of ink papers, glue removal from paper), cellulose produced by *Aspergillus* sp. and *Chaetomium* sp. (hydrolysis of cellulose) and protease produced by *Streptomyces griseus*, *Aspergillus sojae* (removal of animal glue and calcium caseinate) [34, 42].

The enzymatic removal depends on mural paintings in different techniques. In case of frescoes, organic deposits are removed in an easy way because pictorial layer does not contain protein. In case of a secco technique, pictorial layer contains proteins and it should not be destroyed by enzymatic treatment.

The enzymatic removal depends also on the following aspects: the nature of the organic medium used in the painted layer, the type and nature of the organic deposits on the painted surface and the bond between the organic deposits and the painted surface.

The Egyptian artists used some types of organic media such as Arabic gum, animal glue and egg yolk. The experimental studies were conducted on those samples which are similar to those murals by using protease enzyme in the cleaning and removal of bat blood patches. Protease was applied with a soft brush to clean and remove blood from the experimental samples (mock ups). Significant and crucial results were obtained, and then applied in removing the blood patches of bats from the surfaces of archaeological murals both colored and non-colored in some Egyptian archaeological sites [43]. The effectiveness of protease enzyme in the removal of bat blood patches was proved by Atomic Absorption Spectrometry, FTIR Spectroscopy, CHNS Analysis, optical microscopy, Scanning Electron Microscopy and Colour change measurements.

Biocleaning of murals could be also performed with gels and entrapped microbial enzymes [44].

4.2.3. Biocleaning case studies with viable microbial cells and enzymes

The XIV century fresco called Conversione di S. Efisio e battaglia (50 m^2) was detached in the 1980s from the Pisa Camposanto Monumentale (Italy) involving the application *on site* of a wide gauze directly on the fresco surface using animal glue; the back of the fresco was reinforced by a canavas cloth applied using casein [45]. After mechanical cleaning a cotton wool layer soaked with *P. stutzeri* strain A29 suspension was applied onto the fresco surface for 8-12 hours. When the carrier was removed and after washing with distilled water, small organic matter residues still remained on the surface.

The second treatment was applied with protease Type XIX and collagenase Type IA. Enzymes were applied on swab, paper disc and brush. The best results had been obtained using brush because the gentle pressure of the bristles of the brush and repetitive and gentle mechanical pressure of the manual application. During application a portable heating device assured the optimal temperature for enzymatic reaction (38^0C) and the environmental temperature was 28^0C. Local pH was 7,8-8,2 and treatment time was 10-15 min. Total ATP content values, microscopical analysis and test of viability showed the lack of growth. The greatest advantage of bacterial and enzymatic biocleaning is that it is not destructive and removes only organic deposits or altered organic compounds from fresco. *P. stutzeri* strain A29 has non-specific activity and the enzymes are highly specific, but limited to available organic deposit.

4.3. Biological control

In the field of cultural heritage, biological control is based on the inoculation of an antagonist microorganism or to apply some metabolic compounds in order to kill biodeteriogens (during restoration) or to prevent the growth of new biodeteriogens (in the end of restoration). In this area, innovative researches are focused to develop “green biocides” extracted from microbial cultures or plants as well as to develop a methodology for quick identification and selection of lipopeptides-producer strains [46].

Biological control based on microbial biocides shows an increasing interest for cultural heritage due to target specificity, low toxicity and low environmental impact.

There are species of *Bacillus* (*B. subtilis*, B. *amyloliquefaciens*) which synthetize antifungal peptides, antifungal lipopeptides and antimicrobial polypeptides [21]. Silva et al. [46] found that *Bacillus* strains produce biosurfactant lipopeptides that inhibit fungal biodeteriogens (*Cladosporium* spp., *Penicillium* spp, *Fusarium oxisporum* and *Aspergillus niger*). Presently in the Microbial Culture Collection of Institute of Biology there are two *Bacillus* strains able to inhibit the growth of black fungi (yet unpublished results).

Caselli et al. [47] evaluated in vitro the potential antimicrobial activity of a biocompound containing spores of *Bacillus* spp. (*B. subtilis*, *B. pumilus*, and *B. megaterium*).The results showed that *Bacillus*

almost completely inhibited the growth of all microbes isolated from 17th century easel painting attributed to Carlo Bononi due to a non-specific inhibitory action. These results suggest that bacteria belonging to the *Bacillus* genus might be able to counteract *in situ* the growth of biodeteriogens.

5. Concluding remarks

Biodeterioration is a process produced by living organisms on the monuments, stone works frescoes, easel paintings, documents and archaeological objects. Microorganisms can have either a negative action on cultural heritage due to their major role in biodeterioration or a positive one through their metabolites involved in biocleaning. A deep understanding of microbiota colonizing artworks is very important for scientific restoration and for establishing of optimal methods for prevention and conservation. Nowadays, due to the fact that restoration is performed using chemical and physical technologies (not always with satisfactory effects, with potential to damage the artwork, human toxicity and environmental hazards) research is focused on innovative biotechnologies included in the term of biorestoration, understanding microorganisms not only as dangerous but also as potential tools for restoration. Next generation sequencing methodologies are used for the identification of the whole microbiota in real time and to find its composition and modification before and after biocleaning or during bioconsolidation. The bacterial carriers contribute to the efficiency of biocleaning. Promising results had been obtained with agar-gauze gel activated with bacteria for biocleaning of mural painting.

For the future, a better information of conservators and restorers in the use of the biotechnologies applied to restoration and conservation, together with new results on optimizing the biocleaning and bioconsolidation about microorganisms, enzymes and bioactive molecules will contribute to their technological transfer on a large scale.

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