

1 **Back to the past. Forever young: cutting-edge biochemical and**  
2 **microbiological tools for cultural heritage conservation**

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19 ***ABSTRACT***

20 Ancient documents and milestones of human history such as manuscripts and textiles are  
21 fragile and during aging undergo chemical, physical and biological deterioration. Among the different  
22 causes of damage, also human intervention plays a role since some restoration strategies proved to be  
23 transient and/or they generated further damage. Outdoor monuments undergo deterioration since they  
24 are exposed to pollution, weathering, microbial attack (giving rise to undesired pigmentation,  
25 discoloration or true dissolution, corrosion, and overall decay) as well as men-made damage (i.e.  
26 graffiti). This review article reports the best fitting strategies used to restore wall paintings, outdoor  
27 monuments, textiles and paper documents to their ancient beauty by employing “soft” bio-based  
28 approaches such as viable bacteria or suitable enzymes.

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31 **Key-words:** immobilized enzymes, biocleaning, caseinase, collagenase, viable bacteria, graffiti,  
32 bioconsolidation

33

## 34 **INTRODUCTION**

35 Artworks may undergo a number of degradation and deterioration events, which widely vary  
36 depending on the specific artifact and the environment and conditions of conservation. These  
37 parameters may be extremely different if we consider, for instance, a book conserved at controlled  
38 temperature and humidity in a library or in a museum, or a stone statue or a cathedral, which are  
39 constantly exposed to weathering, pollution, microbial colonization (extensively reviewed in Mazzoli  
40 et al. 2018), vandalism acts, etc... It is worth reminding that damaging of artworks is sometimes the  
41 effect of previous restoration interventions which underwent deterioration themselves during time, as  
42 in the case of glues applied to consolidate wall paintings or ancient textiles (Beutel et al. 2002; Ferrari  
43 et al. 2017).

44 Cleaning and/or restoration of artworks by biotechnological approach has been performed by  
45 using enzymes or microorganisms or a combination of both strategies, depending on the specific  
46 artifact and issue (Figs. 1, 2; Table 1). Bio-based methods have a number of advantages over more  
47 consolidated techniques for artwork restoration (such as those using non-aqueous solvents,  
48 bleaching and mechanical treatments), because of their lower impact on the environment, reduced  
49 toxicity for operators and higher selectivity and safety for artworks themselves (Barbabetola et al.  
50 2016). Enzymes are generally characterized by extremely high substrate specificity which allows high  
51 selective choice depending on the “damaging material” (e.g. proteins, polysaccharides, lipids) to  
52 treat/remove. Moreover, enzymes can be chosen whose catalytic activity is optimal at the most  
53 suitable pH/T ranges for treating a certain artwork thus reducing the application time (Germinario et  
54 al. 2017). Enzymes have been used in aqueous formulations, with or without a gel as sorbent, and in  
55 ionic liquids (Hrdlickova Kuckova et al. 2014). On the other hand, the use of enzymes may be limiting  
56 because of the relatively high amounts required, their relatively high cost, the need for controlled  
57 application conditions (e.g. pH and T) and for skilled operators (Barbabetola et al. 2016). Therefore,  
58 the use of microorganisms has sometimes been preferred, for instance when very resistant or complex  
59 deposit materials (i.e. mixture of heterogeneous substances) or very extended surfaces (e.g. the  
60 surface of a cathedral) needed to be removed/treated. In addition, the use of living microorganisms is  
61 necessary in the case of complex phenomena such as calcium carbonate deposition for the  
62 bioconsolidation of stone material (Dhami et al. 2014). As compared to enzymatic strategies, the use  
63 of living microorganisms for biocleaning of artworks is certainly less expensive and may require less  
64 controlled environmental conditions, although it is generally less selective (Webster and May 2006).

65

66 *Biocleaning-biorestitution of paper documents and textiles*

67 One of the most common causes of biodeterioration of ancient papers (e.g. books, documents)  
68 and textiles that are preserved in museums, libraries and archives are glues employed, with specific  
69 variations and modifications, to manufacturing or consolidating/restoring these artifacts  
70 (Barbabetola et al. 2016; Ferrari et al. 2017). In the case of paper artworks, glues have been used in  
71 manufacturing, such as for bonding and lining of prints, drawings, documents, which were mounted  
72 (partly or completely) on secondary support by means of glue spots, as well as for restoration  
73 (Barbabetola et al. 2016). As regards historical or ethnographic textiles, glues have mainly been used  
74 for restoration purposes, e.g. to fasten them to textiles or to solid (paper or wood) supports (Ahmed  
75 and Kolisis 2011). In the past, glues of either vegetal (i.e. starch) or animal (i.e. collagen and/or  
76 casein) origin have been used for these purposes (Barbabetola et al. 2016; Ferrari et al. 2017). Both  
77 animal and vegetal glues are made up of natural polymers, that is mainly proteins (i.e. collagen  
78 derived from the bones, skins, tendons and cartilage of mammals or fish swimming bladder) or  
79 polysaccharides (i.e. amylose and amylopectin derived from different plants, such as potato, rice,  
80 corn or wheat), respectively (Barbabetola et al. 2016; Ferrari et al. 2017). Through aging, these glues  
81 undergo stiffening and thickening which may in turn generate distortions, tensions and discoloration,  
82 or form intricate layers that are very recalcitrant to being removed (Blüher et al. 1995; Gostling 1989).  
83 In the case of animal glues, humidity, temperature, UV radiation and pollutants can generate protein  
84 cross-linking and/or hydrolysis/oxidation of peptide bonds, while microbial metabolism produces  
85 acid molecules and pigmented spots (Barbabetola et al. 2016). Starch glue has been commonly used  
86 for ancient textile restoration (Ahmed and Kolisis 2011; Whaap 2007). After aging, starch paste is  
87 generally found in shrunk, cracked, rigid and brittle form, which cannot provide enough adhesion for  
88 effective support. In this form, it can cause heavy damage to ancient textiles because of concomitant  
89 embrittlement, hardening, yellowness and acidity of the latter. Furthermore, starch may be a source  
90 of contamination by amylolytic fungi and bacteria that contribute to textile decay over time, especially  
91 when a suitable degree of humidity supporting microbial growth is present.

92 Consequently, cleaning of glue residues is often a priority in the restoration of ancient paper  
93 or textile artworks. Current mechanical and chemical methods display serious drawbacks mainly  
94 related to aggressiveness towards material and/or toxicity for the restorers and/or the environment.  
95 Humidification (also called wet-cleaning) has been used to swell starch paste, however it generally  
96 needs long treatments which are unsuitable for paper or textile artifacts and is often insufficient for  
97 aged or hardened glues. Bio-based methods, i.e. the use of enzymes or microorganisms, have been  
98 shown to be a very efficient alternative in a number of cases (Ahmed and Kolisis 2011; Barbabetola  
99 et al. 2016; Ferrari et al. 2017).

100 Enzymes are certainly the most frequently used method for the treatment of glue-damaged  
101 paper (Banik et al. 2003; Corbi et al. 2005; DeSantis 1983; Sandrine 2002) or textiles (mainly linen,  
102 silk and cotton fabrics, so far) (Ahmed and Kolisis 2011; Ciatti et al. 2010) and several successful  
103 examples have been reported in the literature. For instance, trypsin has been used for detaching a  
104 compact block of leaves (Wendelbo 1976). Amylases and proteases have been employed for  
105 detaching graphics from their backings (De la Chapelle 2003; Segal and Cooper 1977). Very recently  
106 enzyme extracts with protease activity isolated from marine invertebrates have been used to remove  
107 aged/altered protein glue layers from the velinatura (Japanese paper bonded by animal glue) of  
108 ancient oil on canvas or from polychrome wood (Palla et al. 2016). This proved to be a cutting-edge  
109 strategy also useful to bio-clean fragile artworks such as wax sculptures. An additional advantage of  
110 these marine invertebrate-derived extracts is their antimicrobial activity useful to control  
111 bacteria/fungi growth (Palla and Barresi 2017). It is worth noting that application of enzymes in  
112 solution is not always suitable for paper or textile artworks, since it may involve artifact flooding with  
113 excess water which favors mold and fungi colonization and growth and thus causes further damage  
114 to the artifact (Ahmed and Kolisis 2011). Actually, water-dissolved  $\alpha$ -amylase preparations have been  
115 applied locally either in solution (Ahmed and Kolisis 2011) (Fig. 1a-c; Table 1) or as poultice (Bott  
116 1990; Chapman 1986; Shibayama and Eastop 1996) for the bio-restoration of starch-glue treated  
117 textiles. In general, the use of immobilized enzymes is preferable for these applications. Theoretically,  
118 all enzymes can be immobilized. However, it is worth reminding that the immobilization yield and  
119 the enzyme efficiency should be determined for each specific enzyme and immobilization strategy,  
120 to limit the loss of enzyme and catalytic activity. A ready-to-use poultice of amylolytic enzymes,  
121 called Albertina Kompresse, was developed by an Austrian group for removing non-swellable starch-  
122 based glue from graphic artworks of albums of the “Albertina” graphic collection in Vienna (Schwarz  
123 et al. 1999). Phytigel™ was used for lowering and controlling water content in enzyme solutions  
124 (Iannucelli and Sotgiu 2009) used for cleaning -etchings depicting the China of Clemente VIII,  
125 dating 1598. Gellan hydrogel-immobilized  $\alpha$ -amylases have been developed for removing starch  
126 paste from ancient paper documents (Mazzuca et al. 2014). A gellan-immobilized bacterial  $\alpha$ -amylase  
127 has been recently used to clean a wool shroud dating back to the Coptic period from a starch glue that  
128 had been used in the 1950s to temporary consolidate the textile (Ferrari et al. 2017) (Fig. 1d-h; Table  
129 1). After selection of the suitable enzyme (among those commercially available) and optimization of  
130 the conditions for enzyme immobilization, the cleaning of the back of the two fragments (about 4 m<sup>2</sup>  
131 of textile) composing the tunic was completed in 160 h of work (Ferrari et al. 2017). A recent study  
132 (Barbabetola et al. 2016) has described the first attempt to bio-cleaning ancient paper from animal  
133 glue by using living bacteria (Table 1). To this aim, non-pathogenic, non-spore-forming and non-

134 cellulolytic *Ochrobactrum sp.* TNS15E was used after immobilization on agar gel. This bacterial pack  
135 was used to remove glue layers from paper documents dating back to the 17-18<sup>th</sup> century. Four-hour  
136 treatment was sufficient to clean the cellulose fibers from glue, as confirmed by both colorimetric  
137 and scanning electron microscopy (SEM) analyses.

138           Apart from paper/textile biocleaning from glue, it is worth reminding the case of aged drying-  
139 oil stains on both ancient paper and textiles. During drying and aging, double bonds of unsaturated  
140 fatty acids are oxidized by oxygen in the air, giving rise to multiple products which include  
141 nonhomogeneous polymeric network of triacylglycerides which may be hard to being removed  
142 (Ahmed et al. 2010; Blüher et al. 1997). Lipases such as that of *Candida cylindracea* have been used  
143 to clean such aged drying-oily stains from paper documents (Blüher et al. 1997) or textiles such in  
144 the case of a coptic tunic (Ahmed et al. 2010) (Table 1).

145

#### 146 [Biorestitution/biocleaning of stone artworks](#)

147           Durable stone (e.g. marble and/or limestone) has been used for the construction of a multitude  
148 of artworks and monuments all along the human history and all over the world including the Egyptian  
149 Pyramids, the Greek and Roman temples and theaters, the European Cathedrals and the Taj Mahal in  
150 India. Unfortunately, all of them have been suffering from progressive deterioration caused by both  
151 biotic and abiotic agents (Dhami et al. 2014). These numerous factors have led to stone dissolution,  
152 staining or color alteration, surface alteration, bio-corrosion and transformations into smaller sized  
153 crystals, etc... (Chand and Cameotra 2011). In the recent decades, microbial biofilm production,  
154 deposition of organic (such as residual hydrocarbons and other organic pollutants in dust) and  
155 inorganic compounds (formation of nitrate and sulfate alterations such as the black crusts) have been  
156 among the main deterioration events (Antonioli et al. 2005; Di Pippo et al. 2009; Fernandes 2006;  
157 Warscheid and Braams 2000). Actually, limestone mainly consists of the most stable polymorph of  
158 calcium carbonate, i.e. calcite, (with only a small content of aragonite) but is very porous and  
159 hydrophilic. This makes limestone very susceptible to water flush (especially acid rain),  
160 environmental pollutants and physical, chemical and biological (e.g. microorganisms) weathering  
161 (Dhami et al. 2014). Therefore, survival of many cultural and historical assets is in threat. One of  
162 such examples is the cave of Lascaux in southwest France which is considered the best conserved  
163 prehistorical example of human wall painting art (they are also named the paleolytic Cappella  
164 Sistina). In this site, infection of *Fusarium sp.* and other molds have deteriorated the floor and banks  
165 of the main chamber (Rosenbaum 2006), but also autotrophic organisms such as green algae have  
166 produced green pigments because of the intense illumination and improved CO<sub>2</sub> availability related

167 to visitors (Bastian et al. 2010). Martin-Sanchez and co-workers (2012) have extensively studied the  
168 effectiveness of biocides in the cave biocleaning. Fungicides had been intensively applied to treating  
169 the cave since 2001 that were essentially targeted to remove *Fusarium sp* but obtained little success.  
170 In 2008, a new biocide treatment was planned due to black stains that appeared on the cave surfaces.  
171 DGGE analysis on these stains showed the presence of *Ochroconis lascauxensis*. This result  
172 demonstrated the ineffectiveness of the previous biocide treatments on the long time which appeared  
173 to favor colonization by other fungal strains and therefore increase fungal diversity. Later, i.e. in  
174 2010, fungal communities were quite different from those detected in 2008, since the main identified  
175 strain was a yeast belonging to the *Herpotrichiellaceae* family. It is clear that careful preliminary  
176 study on the possible advantages and disadvantages of applying biocides in subterranean  
177 environments is required (Martin-Sanchez et al. 2012).

178 Many attempts have been made to fix such structural damages by application of traditional  
179 conservative treatments such as organic and inorganic chemicals (Lazzarini and Laurenzi Tabasso  
180 1986). However, these agents have been most often low effective, in spite of their aggressiveness  
181 (which, on the other side, has led the concomitant risk of further damaging the artwork). Moreover,  
182 these strategies involve the use of high amounts of solvents, which are finally discarded in the  
183 environment creating problems of sustainability (Dhami et al. 2014). Alternatively, physical  
184 treatments such as laser cleaning have been used, but at significantly higher costs (Germinario et al.  
185 2017). Furthermore, all these treatments have short duration effects thus requiring repeated  
186 interventions with relevant economic issues for public and private conservation agencies. Overall,  
187 conventional treatment methods have therefore proved to be unsatisfactory.

188 The shortcomings of conventional strategies have encouraged research in new conservation  
189 and remediation strategies based on biological methods (Fernandes 2006). As for the treatment of  
190 other type of artifacts, bio-based restoration approaches for stone materials are characterized by lower  
191 cost, toxicity and aggressiveness towards the artworks (Germinario et al. 2017). As described in the  
192 following sections, bio-based methods have been used to remove different degradation products from  
193 stone monuments, wall paintings, and marble statues (Germinario et al. 2017), including deposits of  
194 environmental pollutants (Margesin et al. 2011) and synthetic polymers present in adhesives  
195 (Giordano et al. 2018) as well as in paints used by graffiti writers (Sanmartin et al. 2014) (Fig. 2;  
196 Table 1). In addition, biocleaning has been performed on stone artworks suffering from inaccurate or  
197 aged restoration intervention (Beutel et al. 2002; Antonioli et al. 2005) (Fig. 2; Table 1).

198

199 Removal of sulfate and nitrate alterations

200 One of the most important causes of decay of calcareous stones is the conversion of calcium  
201 carbonate into calcium sulfate (gypsum) mainly caused by acid rains (i.e. containing significant  
202 amounts of sulfuric and nitric acid) (Ranalli et al. 1997). For instance, the genesis of “gypsum crusts”  
203 on the surface of such porous material can engender following fractures of the underlying stone.  
204 When calcium sulfate salts are accumulated together with atmospheric particles (pollen, dust, spores,  
205 small particles of smog) the so called “black crusts” are formed (Fig. 2a). For the removal of sulfates  
206 from artistic stoneworks, procedures based on the use of sulfate-reducing bacteria have been reported.  
207 Different bacterial strains of the genus *Desulfovibrio* (e.g. *D. desulfuricans* and *D. vulgaris*) (either  
208 pure or in mixed cultures) have been applied under anaerobic conditions to marble samples directly  
209 or after adhesion to a sepiolite matrix (Ranalli et al. 1997). The use of sepiolite promoted sulfate  
210 removal on both simulated samples and real marble statue artifacts. On the latter, 81 % sulfate  
211 removal was obtained after 36 h treatment (Ranalli et al. 1997). Actually, *D. desulfuricans* (Ranalli  
212 et al. 1997) and *D. vulgaris* (Ranalli et al. 1997; Cappitelli et al. 2007a; Alfano et al. 2011) have been  
213 widely employed in restoration/removal of sulfate crusts from other artifacts (Table 1). Use of  
214 biotechnological cleaning on durable stone monuments can sometimes comply with multiple types  
215 of deterioration such as described by Cappitelli et al. (2007b). In this study, the sulfate-reducing  
216 bacterium *D. vulgaris subsp. vulgaris* ATCC 29579 was employed to remove the black crust found  
217 on marble of the Milan Cathedral (Italy). Compared to chemical cleaning (i.e. ammonium carbonate-  
218 EDTA) strategy, the microbial-catalyzed approach resulted in more homogeneous removal of the  
219 deposits and higher preservation of the original surface (Cappitelli et al. 2007b) (Table 1). Both  
220 chemical and biological treatments converted gypsum (i.e. calcium sulfate) to calcite (i.e. calcium  
221 carbonate), allowing consolidation. However, the chemical strategy also formed undesirable sodium  
222 sulfate while the use of *D. vulgaris* did not (Cappitelli et al. 2007b). Nonetheless, biological removal  
223 of sulfates may require quite long application periods, depending on the thickness of the crust. A  
224 recent study has demonstrated that this period can be greatly shortened and general efficiency of  
225 biocleaning can be significantly improved by combining the use of sulfate-reducing bacteria with a  
226 non-biological strategy, e.g. the use of a non-ionic detergent (Troiano et al. 2013) (Fig. 2a, b; Table  
227 1). This combined strategy shortened application times of about 38-70 % depending on the specific  
228 artifact to be cleaned (Troiano et al. 2013).

229 Another consequence of acid rains (and of the action of living microorganisms) is the deposit  
230 of calcium nitrate salts on stone buildings and wall paintings (Dhami et al. 2014). Here, again,  
231 pollution increases the presence of various nitrogen oxides in the atmosphere that in turn may react  
232 with rain water and form nitrous and, more abundantly, nitric acid which then reacts with stone and  
233 replaces calcium carbonate with calcium nitrate (Dhami et al. 2014). Different strains of



234 *Pseudomonas spp.* have been recently applied for removing calcium nitrate salts from two stone  
 235 monuments. Agar-entrapped *Pseudomonas stutzeri* DSMZ 5190 has been used for the biocleaning of  
 236 nitrate efflorescence from wall paintings located in the lunettes of the central vault of the Santos  
 237 Juanes church in Valencia, Spain (Bosch-Roig et al. 2013) (Fig. 2c-e; Table 1). The chosen strategy  
 238 proved to be extremely efficient allowing to remove 92 % of the precipitates in 90 minutes.  
 239 *Pseudomonas pseudocaligenes* KF707 has been used to remove nitrate salts from the tuff stone  
 240 surfaces of the 12<sup>th</sup> century Matera Cathedral, Italy (Alfano et al. 2011) (Table 1). Here, carbogel-  
 241 entrapped bacteria were applied to the Cathedral walls and allowed quick removal of the surface  
 242 nitrate deposits, since 55 % of the nitrate salts were “cleaned” after 24 h.

243

#### 244 Bioconsolidation

245 Apart from interventions aimed at removing superficial deposits and/or crust from stone  
 246 monuments, the use of calcifying bacteria offers a chance to consolidate decayed building structures  
 247 and materials. This application, sometimes also called microbial geotechnology (intending microbial-  
 248 based technology for civil structures) actually mimics nature since many carbonate rocks have been  
 249 cemented by carbonate precipitation induced by microorganisms during Earth geological cycles. This  
 250 relatively novel and environmental-friendly technology has been studied for at least 20 years and has  
 251 already been used for protecting and/or restoring different decayed construction materials/artifacts  
 252 (Dhami et al. 2012; 2013).

253 Calcium carbonate precipitation is a chemical process (described by equation 1) which is  
 254 influenced by four main factors, i.e. calcium concentration, amount of dissolved inorganic carbon  
 255 (DIC), availability of nucleation sites and pH (Hammes and Verstraete 2002).



257 
$$\text{Eq. 2} \quad K_{SP, \text{Calcite}, 25^\circ\text{C}} = [\text{Ca}^{2+}] [\text{CO}_3^{2-}] = 4.8 \times 10^{-9}$$

258 Calcium carbonate precipitation occurs when the product of concentrations of  $\text{Ca}^{2+}$  and  $\text{CO}_3^{2-}$  is  
 259 higher than the solubility product ( $K_{SP}$ ) of calcium carbonate (Eq. 2). However, the amounts of  $\text{CO}_3^{2-}$   
 260 in a given system depends on both the amount of DIC (which in turn depends on several parameters  
 261 such as temperature and partial pressure of carbon dioxide) and pH. Because of the high number of  
 262 parameters that may contribute to control calcium carbonate precipitation, different bacteria, isolated  
 263 from different habitats, are able to create local micro-environments that induce such phenomenon  
 264 (Hamilton 2003) (Fig. 3). The four main groups of microorganisms that may influence calcification  
 265 are: (i) photosynthetic organisms such as cyanobacteria and algae, (ii) sulfate reducing bacteria

266 responsible for dissimilatory reduction of sulfates, (iii) organisms utilizing organic acids, and (iv)  
267 organisms that are involved in nitrogen cycle either by ammonification of amino acids/nitrate  
268 reduction or hydrolysis of urea (Stocks-Fischer et al. 1999; Hammes and Verstraete 2002; Jargeat et  
269 al. 2003) (For an exhaustive review please refer to the study of Dhami et al. 2014). The precipitation  
270 of carbonates by bacteria through urea hydrolysis is the most straightforward and easily controlled  
271 mechanism of microbial induced calcium carbonate precipitation since it produces high amounts of  
272 carbonates and an alkaline environment (Dhami et al. 2014). Boquet et al. (1973) firstly demonstrated  
273 the precipitation of calcium carbonate by soil bacteria under laboratory conditions (Fig. 3). At that  
274 time, several *Bacillus spp.* and *Pseudomonas aeruginosa* were shown to form calcite crystals. In  
275 1990, Adolphe et al. patented the concept of using calcifying microorganisms to treat artificial  
276 surfaces and founded the “Calcite Bioconcept” company. However, the first *in situ* application of  
277 bioconsolidation was carried out in Thouars (France) on the tower of the Saint Médard Church by  
278 using *Bacillus cereus* only in 1993 (Le Metayer-Levrel et al. 1999) (Table 1). Although this  
279 application was judged as successful, some drawbacks were: the need to regularly repeat the treatment  
280 (for instance, each 10 years); the presence of natural pigments in the nutritional medium of *B. cereus*  
281 which co-precipitated with calcium carbonate thus giving the new stone layer a light persistent  
282 coloring; the formation of endospores and a thin biofilm of *Bacillus sp.* For these reasons, Rodriguez-  
283 Navarro et al. (2003) proposed to replace *Bacillus sp.* with a Gram-negative, non-pathogenic soil  
284 bacterium, i.e. *Myxococcus xanthus*. Tiano et al. (1999) studied the effect of *Micrococcus spp.* and  
285 *Bacillus subtilis* on Pietra di Lecce bioclastic limestone. Variations in the kind of bacteria used and  
286 the methods for bacterial cell delivery to the stone surface have been tested by different Authors with  
287 variable success (Daskalakis et al. 2014; Dhami et al. 2014; Helmi et al. 2016; Micallef et al. 2016).  
288 Recently, the use of indigenous calcifying bacteria for re-inoculation of stone monuments has been  
289 proposed as an alternative strategy for bioconsolidation (Jroundi et al. 2017). However,  
290 microbiologically driven calcification remains more complex than chemical methods, since microbial  
291 activity depends on many factors such as temperature, pH, concentrations of donors and acceptors of  
292 electrons and concentration and diffusion rates of nutrients and catabolites. Hence, the use of  
293 microbial calcification at large scales has not been always encouraged since it may be hard to manage  
294 (Dhami et at. 2014). Also the cost of media required for bacterial growth may be a significant  
295 economic limit of this approach (Achal et al. 2009; 2010).

296

297 *Biorestitution of wall paintings*

298 As previously mentioned, also stone artworks may suffer from inaccurate or aged restoration  
299 strategies. This was the case of two wall paintings covered by animal glue layers during restoration  
300 interventions which have been restored using different bio-based approaches as described by Beutel  
301 et al. (2002) and Antonioli et al. (2005), respectively (Table 1; Fig. 2). The first study concerns  
302 medieval wall paintings called “Falcon hunt-meeting of the living and the dead” located in St.  
303 Alexander church in Wildeshausen, Germany. These paintings were suffering from severe peeling  
304 off from the roughcast surface (Beutel et al. 2002) (Table 1). Actually, this is a common problem of  
305 wall paintings in medieval churches of the Northern Europe since they have long been treated by  
306 application of casein layers to stabilize them (Beutel et al. 2002). As for other glue-like matrices,  
307 aging in addition to climate effects causes progressive hardening and stiffening of casein layers thus  
308 causing an even more drastic peeling off of the painted parts from the surface (Beutel et al. 2002).  
309 This problem was fixed by removing aged casein layers through application of a selected microbial  
310 serine-protease (Alcalase 2.5 DX-L). The enzyme was covalently immobilized onto an epoxide-  
311 functionalized cellulose acetate membrane (Beutel et al. 2002). By 2D fluorescence monitoring of  
312 the tryptophan exposed by casein hydrolysis, it could be estimated that 30-minute treatment was  
313 sufficient for substantial removal of the casein layers from the mural painting.

314 The case of “Conversion of S. Eufisio and battle” fresco by Spinello Aretino at the monumental  
315 Cemetery of Pisa (Italy) was even more complicated (Fig. 2f, g; Table 1). Because of weathering and  
316 other environmental aging, this fresco needed to be restored and for this purpose it was removed from  
317 the wall surface by using the tear-off technique in the 1980s. Firstly, the fresco was covered with a  
318 gauze that was stucked by applying an animal glue (mixed with high concentrations of formaldehyde,  
319 as antimicrobial agent). Once the glue had been hardened, the fresco was detached from the wall.  
320 Unfortunately, the fresco was then forgotten until 2000s in a storeroom, so that, traditional application  
321 of protease mixtures were unable to remove the gauze (Antonioli et al. 2005). Because of the long  
322 time of storage, it is likely that the presence of formaldehyde had promoted the formation of a resistant  
323 net of cross-linked proteinaceous material, which was very recalcitrant to protease catalysis.  
324 However, the selection of a bacterial strain (i.e. *Pseudomonas stutzeri*) able to grow on chips of  
325 insoluble glue harvested from the “clothed” fresco (and hence possessing the right collagenase  
326 enzymes), finally helped to solve the problem. Actually, it was then possible to use this bacterial  
327 strain (i.e. cotton strips impregnated with live *Pseudomonas stutzeri* were applied) directly on the  
328 fresco and completely degrade the glue layer and remove the cloth from the fresco after only 12 hour  
329 treatment (Fig. 2f, g).

330

331 *Biocleaning of graffiti and synthetic adhesives*

332 Stone monuments are not only aggressed by atmospheric events or microorganisms but,  
333 unfortunately, also by vandalism acts, including painting by unauthorized graffiti. Graffiti materials  
334 have a complex chemical composition that comprises synthetic polymers such as acrylics, alkyds and  
335 nitrocellulose, and several additives (Germinario et al. 2017). Quick removal of graffiti is an  
336 important issue, since the fresher the graffiti, the easier is their removal. Here again, bio-based  
337 (through enzymes or microorganisms) removal of synthetic materials is an emerging strategy which  
338 has already shown good results in a number of cases.

339 One of the first examples of acrylic material removal by means of bio-based approach has  
340 been described by Bellucci et al. (1999). Here, a lipase has been used to eliminate aged acrylic  
341 ParaloidB72 resin from a 15<sup>th</sup> century tempera painting on panel and a 19<sup>th</sup> century oil painting on  
342 canvas. In both artworks, the presence of surface layers containing ParaloidB72 was the result of  
343 previous restoration interventions occurred in the early 1970s and in the 1980s, respectively (Bellucci  
344 et al. 1999). Cleaning was likely achieved via hydrolysis of the ester groups of the acrylate and  
345 methacrylate units contained in the synthetic resin leading to free carboxylic acid groups. This  
346 reaction therefore generated more hydrophilic products which facilitated acrylic resin removal by  
347 aqueous cleaning systems (Bellucci et al. 1999). Bio-based removal of acrylic materials is particularly  
348 advantageous for treating painting where the use of traditional methods, e.g. organic solvents, would  
349 likely remove also original paint layers. However, the same approach can be employed also to treat  
350 stone materials. For instance, Germinario et al. (2017) tested different lipases in oil-in-water micro-  
351 emulsions for the removal of acrylic marker pen inks from unglazed ceramic surfaces. Very recently,  
352 Palla and co-workers (2017) successfully detached a canvas layer glued by the same acrylic  
353 ParaloidB72 resin to a mosaic sample by applying a gelled (3% Klucel G) enzymatic solution with  
354 esterase activity. The bio-removal was very fast, only 40 minutes at room temperature (Palla et al.  
355 2017). The same research group also reported the removal of adhesive-tape glue residues present on  
356 specific areas of an acrylic paint on canvas. They used a microemulsion of Velvesil Plus® (a  
357 surfactant used in the cosmetic field) containing an esterase derived by a marine organism that proved  
358 to be active even at a temperature lower than 30°C (whereas most commercially available enzymes  
359 have an optimum temperature of 37°C) (Palla et al. 2016). These authors demonstrated that the  
360 enzymatic solution can be merged into the Velvesil Plus® gel (without any negative effect on enzyme  
361 activity) and easily applied to remove the undesired layers. The contact between enzyme and the layer  
362 to be removed was obtained by gently moving the microemulsion, by a soft-brush for 5 minutes  
363 (Giordano et al. 2018). As regards the use of living microorganisms, *D. desulfuricans* has proved able

364 to degrade nitrocellulose-based paints (Giacomucci et al. 2012), while the use of different bacterial  
365 strains has been tested for the bio-cleaning of acrylic polymers used in the restoration field (Troiano  
366 et al. 2014).

## 367 **CONCLUSION**

368 This report demonstrates that it is possible to face cultural heritage damage due to aging,  
369 weathering, pollution or wrong restoration interventions by using bacteria or purified enzymes  
370 suitably immobilized to contain the risk of employing aqueous solutions. Among the wide range of  
371 enzymes commercially available those displaying a good catalytic activity at low temperatures (lower  
372 than 30°C) are very promising since they can be applied also to fragile items. The microbial world as  
373 well as the marine environment seem to be good candidates to be explored for finding such enzymes.  
374 This field is promising also to find, in the future, a solution to contain microbial deterioration (Mazzoli  
375 et al. 2018) thus avoiding the use of acids, solvents and surfactants (dangerous for the artworks, the  
376 art restorers and the environment) for instance by using enzyme- or bacteriocin-mediated bacterial  
377 competition. In this case, the safety and effectiveness of the microorganisms employed is mandatory  
378 and the need for control and analysis before and after treatments strongly recommended.  
379 Interdisciplinary approaches and collaborations between art conservators and biotechnologists,  
380 biochemists and microbiologists is the essential requisite to preserve objects that state the immense  
381 creativity of artists and the high value of human history.

382

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388

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- 560

## Figure Legends

Fig. 1. Biocleaning of ancient textiles from starch glue. Pictures refer to biocleaning of a historical carpet dating back to the Ottoman period and exhibited in the museum of the Faculty of Applied Arts, Helwan University, Egypt (a, b, c) (modified from Ahmed and Kolisis 2011) and a coptic tunic dating back to the 5-6<sup>th</sup> century A.D. and exposed at the Egyptian Museum, Turin, Italy (d, e, f, g, h) (modified from Ferrari et al. 2017). b, c Detail of the carpet before (b) and after (c) the  $\alpha$ -amylase treatment. e-h, Details of the Coptic tunic before (e, g) and after (f, h) the  $\alpha$ -amylase treatment.

Fig. 2. Biorestitution of stone monuments. a, b, Detail of a marble statue dedicate in 1921 by Lina Arpesani to the poetess Anna Zuccari and located in the Monumental Cemetery of Milan (Italy). The black crusts (a) affecting the statue were cleaned (b) by using sulfate-reducing *Desulfovibrio vulgaris* (modified from Troiano et al. 2013). c, d, e Cleaning of the wall painting in the lunette of the Santos Juanes church, Valencia, Spain from nitrate salt efflorescence by means of agar gel-entrapped *Pseudomonas stutzeri*. Pictures represent the fresco area before (c), during (d) and after biocleaning (modified from Bosch-Roig et al. 2013). f, e biorestitution of the Spinello Aretino fresco “Conversion of S. Efsio and battle” in the Monumental Cemetery of Pisa (Italy). f For animal glue removal. Cotton strips impregnated with live *Pseudomonas stutzeri* were applied leading to fresco biocleaning (g) (modified from Antonioli et al. 2005)

Fig. 3. Calcifying bacteria. Colonies of 6 different strains of *Bacillus sphaericus* and *Bacillus lentus* on agar plates during calcium carbonate deposition are shown (Dick et al. 2006).

Table 1. Some of the most significant examples of bioremediation/biocleaning of artworks described in the present study.

Type of artwork	Specific artwork (specimen)	Historical period of the specimen	Issue	Bioremediation/biocleaning strategy	Reference
Paper	Graphic artworks from albums (Graphic Collection Albertina, Vienna, Austria)	XIX century A.D.	Removal of aged starch glue	Gel-entrapped $\alpha$ -amylase	Schwarz et al. 1999
Paper	Paper documents of the Genoese Republic (Central Institute for Graphic Arts, Rome, Italy)	XVII-XVIII century A.D.	Removal of aged animal glue	Agar-immobilized <i>Ochrobactrum sp.</i> TNS15E	Barbabetola et al. 2016
Textile	Coptic tunic (Greek-Roman Museum, Alexandria, Egypt)		Removal of aged oily stains	Lipase from <i>Candida cylindracea</i>	Ahmed et al. 2010
Textile	Carpet (Museum of the Faculty of Applied Arts, Helwan University, Egypt)	Ottoman period	Removal of aged starch glue	$\alpha$ -amylase from <i>Aspergillus oryzae</i>	Ahmed and Kosis 2011
Textile	Coptic tunic (Egyptian Museum, Turin, Italy)	V-VI century A.D.	Removal of aged starch glue	Gellan immobilized $\alpha$ -amylase from <i>Bacillus sp.</i>	Ferrari et al. 2017
Stone monument	Milan Cathedral (Italy)	XV century A.D.	Removal of black crust	Sulfate-reducing <i>Desulfovibrio vulgaris</i> ATCC 29579	Cappitelli et al. 2007b
Stone monument	Florence Cathedral (Italy)	XV century A.D.	Removal of black crust	Carbogel-entrapped sulfate-reducing <i>Desulfovibrio vulgaris</i> ATCC 29579	Gioventù et al. 2011
Stone monument	Matera Cathedral (Italy)	XII century A.D.	Removal of nitrate and sulphate crusts	Carbogel entrapped nitrate-reducing <i>Pseudomonas pseudoalcaligenes</i> KF707 and sulfate-reducing <i>Desulfovibrio vulgaris</i> ATCC 29579	Alfano et al. 2011
Stone monument	Stone column and marble statue, Monumental Cemetery (Milan, Italy)	XX century A.D.	Removal of black crust	<i>Desulfovibrio vulgaris</i> ATCC 29579 plus non-ionic detergent	Troiano et al. 2013

Stone monument	Saint Médard Church, (Thouard, France)	XII century A.D.	Limestone bioconsolidation	<i>Bacillus cereus</i>	Le Metayer et al. 1999
Wall paintings	Wall paintings of the lunettes of the central vault, Santos Juanes church (Valencia, Spain)	XVII-XVIII century A.D.	Removal of calcium nitrate salt efflorescence	Agar-entrapped <i>Pseudomonas stutzeri</i> DSMZ 5190	Bosch-Roig et al. 2013
Wall paintings	Falcon hunt-Meeting of the living and the dead, St. Alexander church (Wildeshauen, Germany)	XIV century A.D.	Removal of aged casein layers	Covalently immobilized protease (Alcalase 2.5 DX-L)	Beutel et al. 2002
Wall paintings	Conversion of S. Efisio and battle by Spinello Aretino, Monumental Cemetery (Pisa, Italy)	XIV century A.D.	Removal of aged formaldehyde-treated animal glue	Cotton strips impregnated with <i>Pseudomonas stutzeri</i> A29	Antonioli et al. 2005
Painting on panel	The Visitation with St. Joseph, St. Zacharias and Four Angels	XV century A.D.	Removal of acrylic resin	Lipase from <i>Candida cylindracea</i>	Bellucci et al. 1999
Painting on canvas	Portrait of a man	XIX century A.D.	Removal of acrylic resin	Lipase from <i>Candida cylindracea</i>	Bellucci et al. 1999

Fig. 1.

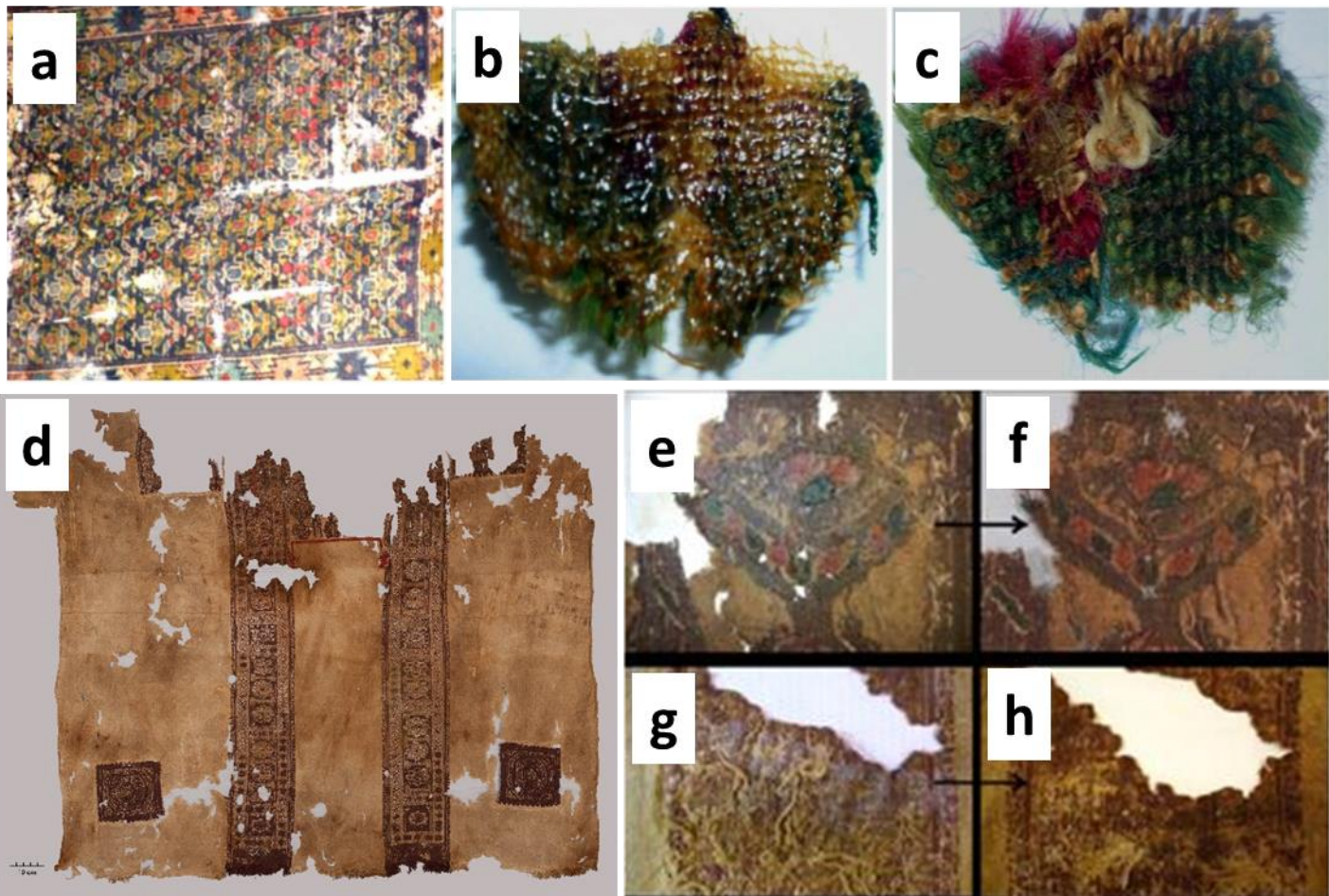




Fig. 2.

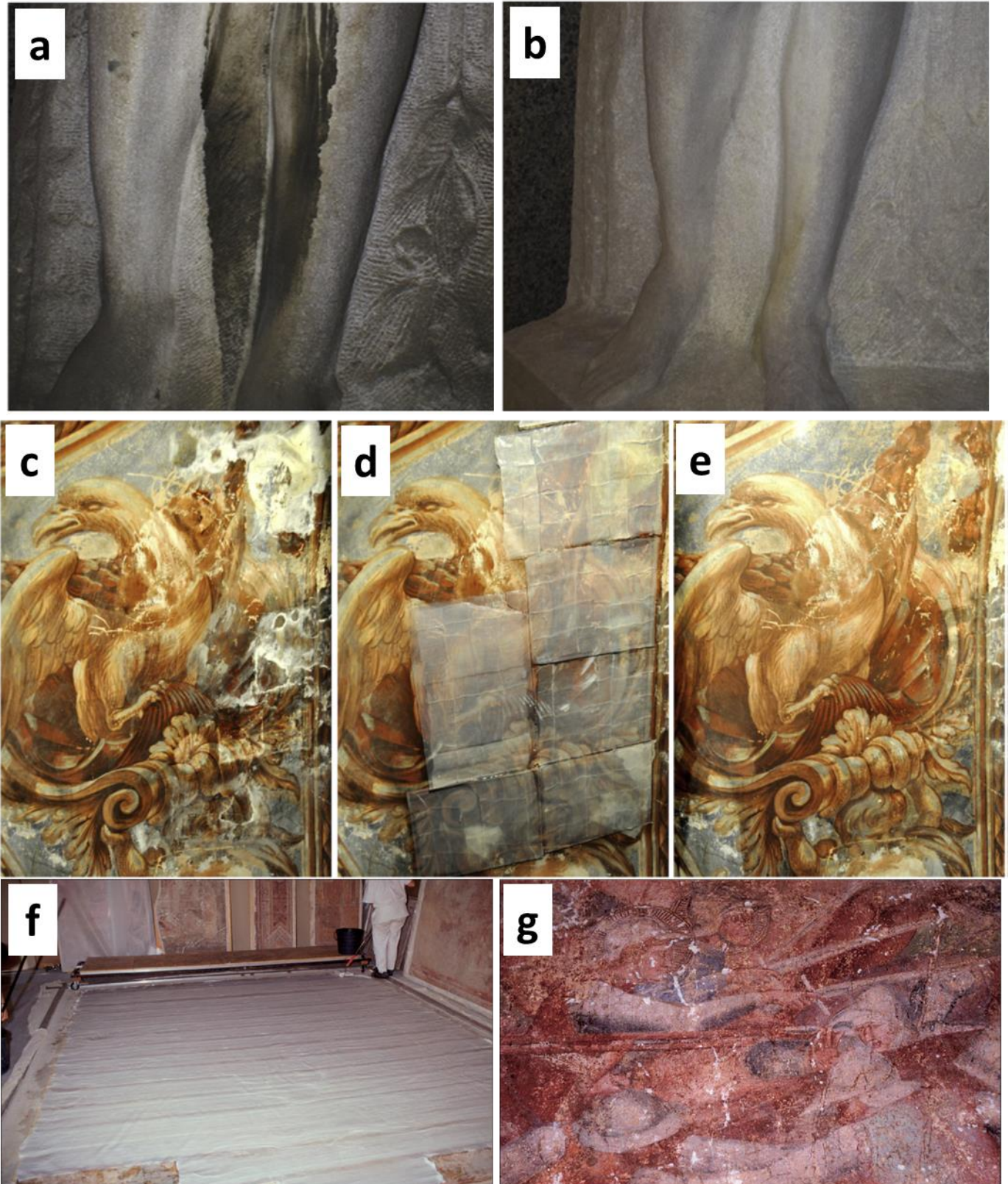
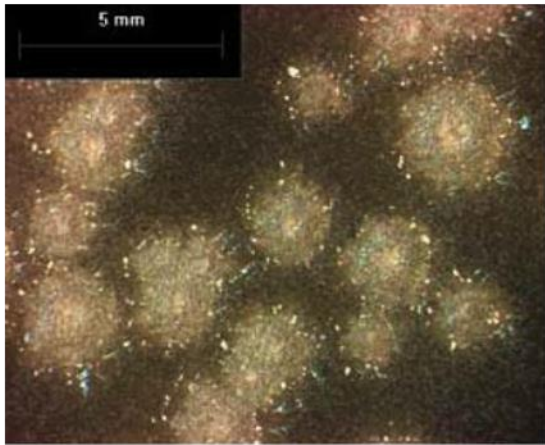
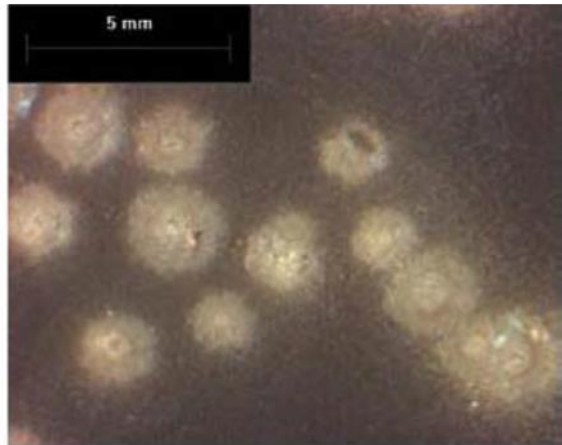




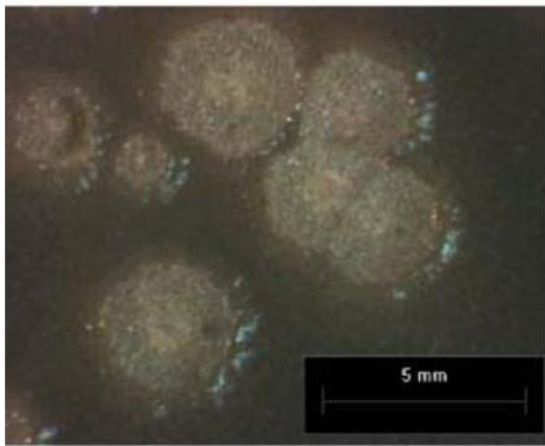
Fig. 3.



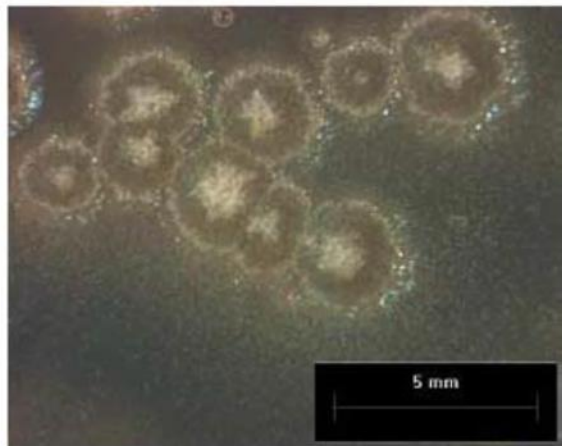
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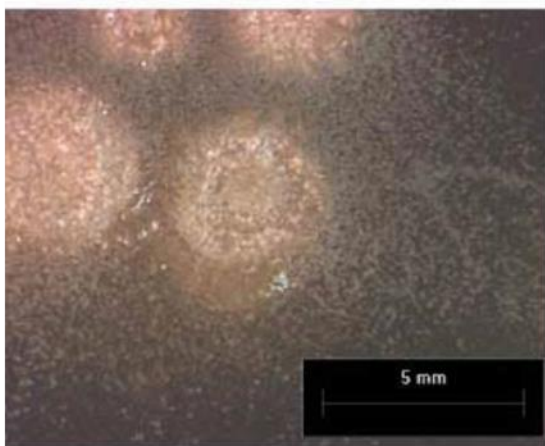
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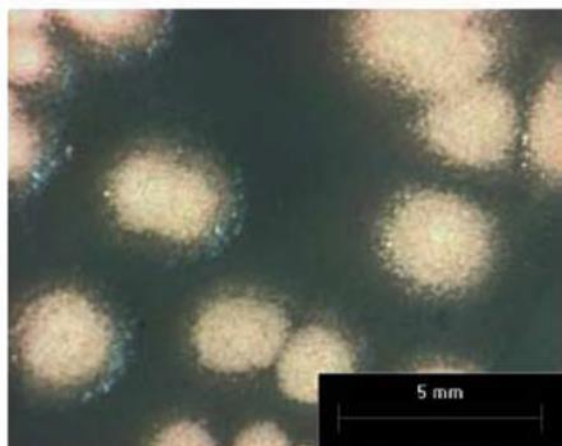
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