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## A safe microbe-based procedure for a gentle removal of aged animal glues from ancient paper

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## ABSTRACT

In the past, the animal glues were used in paper manufacturing and in restoration of artworks preserved in museums, libraries and archives. By ageing they went through deterioration creating distortions, tensions, cockling and discolouration in paper materials. Consequently, the removal of glue residues becomes an essential step in the restoration of ancient and artwork paper material. Current mechanical and chemical methods display serious drawbacks mainly related to aggressiveness towards material or toxicity for restorers. Bio-based methods for paper cleaning rely on the use of enzymes that require skilled operators, optimal application conditions and high costs, creating difficulties in mastering enzyme use so far. This paper describes a first attempt of biocleaning ancient paper from organic deposits using living bacteria. The non-pathogenic, non-spore-forming and non-cellulolytic original strain *Ochrobactrum* sp. TNS15<sup>E</sup> was successfully applied -immobilised in an agar gel-on original paper specimens dating back to the 17th. After 4 h of contact with the bacterial pack, the cellulose fibres underlying glue were disclosed, highlighting the bacterial capacity of removing the glue layer without damaging the paper or leaving undesirable residues. Both colorimetry and SEM analyses proved the results. The procedure is simple, low-cost and safe for the artefact, the restorers and the environment.

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## 1. Introduction

European policy on Cultural Heritage has gradually defined key principles relating to minimum intervention, reversibility and repeatability to promote a more sustainable conservation strategy that implies a change in the research approach as well (European Parliament; Directorate-general for research-2001; European Commission, 2014). The exploration of different routes in different research disciplines is opening new perspectives for innovative applications. Colloid and material science are proposing application for consolidation, cleaning pictorial surfaces, and deacidification of paper, canvas and wood (Baglioni et al., 2013); nanotechnologies are developing application for conservation of papers and parchments (Reina et al., 2014). Biotechnology and especially microbial biotechnology are perfectly suited to this approach and play nowadays a proactive role in searching for new methods both in

diagnostics and restoration.

The controlled use of microorganisms as agents of bio-restoration results in a number of advantages over the use of the consolidated products for restoration: low environmental impact, selectivity for the weathered material, absence of toxic effects for operators, safety for the artwork and cost-effectiveness (Webster and May 2006). The biocleaning of frescoes (Ranalli et al., 2005), the removal of black crusts and nitrate alterations from stone (Ranalli et al., 1997; Cappitelli et al., 2007; Alfano et al., 2011), the bioconsolidation of carbonatic materials (Rodriguez-Navarro et al., 2003; Jroundi et al., 2014; Perito et al., 2014), the biocleaning of hard-to-remove organic and inorganic layered deposits from mural paintings (Mazzoni et al., 2014), represent examples of where microbial technology has already positively contributed. Still remains to assess the range of applicability of this bio-based approach to various set of issues that require improvements over the current techniques and products.

This paper addresses the problem of cleaning paper material from animal glues residues, facing for the first time the biocleaning

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on original organic material, as the membranous artefacts, using living bacteria instead of enzymes.

In the past, animal glues were used in paper manufacturing, for activities of restoration and as adhesive for bonding and lining of prints, drawings, documents, which were mounted partly or completely on a secondary support by means of glue spots. Animal glues are natural polymers, mainly made up of collagen, derived from the bones, skins, tendons and cartilage of mammals (rabbit, ox, etc.) or fish. Animal glue films are highly hygroscopic and go through deterioration by ageing, degradation and biodeterioration. Under fluctuating environmental conditions, the mechanical properties of collagen-based glues are subjected to continual changes, which affect the glue's elasticity, strength and physical stability (Gostling, 1989). Humidity, temperature, UV radiation and pollutants can lead to protein cross-linking, hydrolysis of peptic bonds, oxidation, while the presence of microorganisms produce acid metabolites and pigmented spots, which cause a strong optical-chromatic alteration. As a consequence, yellowing, browning, and embrittlement in the adhesive occur, thus creating distortions, tensions, cockling and discolouration of documents and graphics in the areas where the adhesive was applied (Blüher et al., 1995). So the detachment of the document and the removal of glue staining and residues become an essential step in the restoration and conservation of cultural heritage on paper.

Different cleaning techniques, namely mechanical cleaning, cleaning with non-aqueous solvents, bleaching and washing, have been developed (Crespo and Viñas, 1984), but each method shows drawbacks. Mechanical methods are the most usual and involve the use of brushes, erasers, glass or nylon fibre brushes, and clay powder. These localized methods are too aggressive for the integrity of paper, because they produce holes and tears on the object. Cleaning with non-aqueous solvents consists in the application of dry-cleaning solvents, so called because their volatility causes rapid drying. The most used solvents are hexane, petroleum ether, toluene, chloroform, dichloromethane, acetone, alcohol and petrol. The cleaning power of this type of solvents made their use very widespread among conservators, but it should be remembered that the high number of negative consequences (high toxicity, fire and explosion risks, excessive dehydration of the paper) has limited their employment. Solvent cleaning is probably the most hazardous phase of paper restoration, not only because of the chemicals used, but also because of the working methods that have to be employed: under a fume cupboard with reinforced ventilation that can lead to a too rapid evaporation of solvent impeding from doing a good job (Hey and Petherbridge, 1980).

For many years the most widely used of all restoration techniques was paper bleaching. It was a very popular method, because of its simple method of application and the spectacular results it was able to achieve (Malesic et al., 2008). The most commonly used bleaches are hypochlorites, chloramine T and chloramine B, sodium chlorite, potassium permanganate, and hydrogen peroxide. Bleaching weakens cellulose appreciably, sometimes to the point of complete disintegration, especially in the case of modern documents. Chemical analyses have shown that bleaching is based on oxidation of cellulose, which triggers a process of acidification that breaks up the molecular structure of the cellulose and hence weakens the paper (Crespo and Viñas, 1984). Reductive bleaching treatments (sodium borohydride and tert-butylaminoborane) seems partially to preserve the cellulose (Henniges and Potthast, 2009) and routes for reducing the risks of hydrogen peroxide bleaching in presence of iron ions in paper have been explored (Niehus et al., 2012). Although efforts are made to reduce the danger, bleaching is nowadays considered the most potentially harmful form of cleaning.

Washing for paper cleaning in most cases weakens interfibre

links and decreases inks stability; washing baths must always be coupled with appropriate protective measures to prevent mishaps (Daniels and Kosek, 2004). The action of the water can be augmented with the use of specific substances, such as detergents, colloidal agents or enzymes.

Gellan hydrogel has recently been reported as an alternative tool to the traditional cleaning technique (i.e. immersion in a deionized water bath) (Mazzuca et al., 2014).

Enzymes are certainly today the most used method for the treatment of glue stains on paper. Trypsin has been used for detaching a compact block of leaves (Wendelbo, 1976), amylases and proteases for the detachment of graphics from their backings (Segal and Cooper, 1977; De la Chapelle, 2003). An application of amylolytic enzymes, called Albertina Kompresse, was performed by an Austrian group who developed a ready-for-use enzyme poultice for the easy removal of non-swelling starch-based (amylase) glue (Schwarz et al., 1999); Phytigel™ was used for the reduction and the control of the water content in enzyme solutions (Iannucelli and Sotgiu, 2009) applied for the cleaning of etchings depicting the China of Clemente VIII, dating 1598. Despite the several interesting applications that have been proposed, the restrictive operational conditions required (high temperature, stable pH conditions, favourable saline concentrations) and the high costs have created difficulties in mastering enzyme use so far. Therefore an effort to develop a fully satisfactory solution is welcome.

As a result of the research described in this article, a method based on the use of viable bacterial cells is presented. The procedure does not require restrictive operating conditions and combines the efficacy of treatment with safety, ease and feasibility. Related literature does not offer similar case studies so far, thus this case study shall represent the first attempt to clean glue deposits on paper material through the use of viable bacterial cells.

## 2. Materials and methods

### 2.1. Paper specimens

The application of viable bacterial cells for glue bio-removal was performed on original paper sheets, which were back linings of prints, detached during restoration operations and kindly supplied by the Restoration Laboratory for Works of Art on Paper - Central Institute for Graphic Arts (ICG) in Rome. Samples belong to a nucleus of prints of the 17<sup>th</sup> century. The paper specimens were probably produced by a Northern Italy paper mill and show a watermark, representing the symbol of the Genoese Republic,

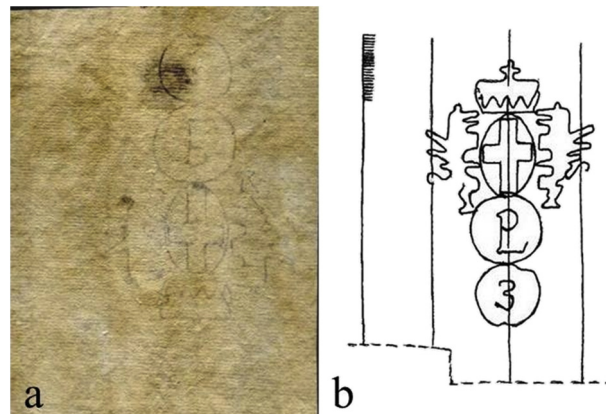


Fig. 1. Paper material used for the glue bio-removal trial showing a watermark, which represents the symbol of the Genoese Republic (a) and a drawing of the watermark (b).

which allowed to date the paper around 1723 (Fig. 1). Paper sheets were cut into 50 pieces of about  $3 \times 2$  cm on which the biocleaning application was performed. Each treatment (including the untreated control) was performed on 10 specimens.

## 2.2. Glue substrates

Two types of animal glues, Cervione glue and Rabbit Skin glue (Antichità Belsito S.r.l., Rome, Italy), were employed in the degradation trials, because they were widely used in the conservation of cultural heritage. Solutions of the two target substrates were prepared at  $10\% \text{ w v}^{-1}$ , according to the following procedure: granules of the two animal glues were first immersed in cold water overnight and then heated in a water bath until complete dissolution. The solutions were then autoclaved at  $121^\circ\text{C}$  for 15 min.

## 2.3. Screening for the selection of glue-degrader strains

The screening for bacterial strains capable to oxidise the target substrates was performed on seventeen strains (Table 1), previously isolated from polluted sites (Sprocati et al., 2006), industrial wastewaters and archaeological sites (Sprocati et al., 2008) and maintained in the ENEA-Lilith microbial culture collection. The screening was performed by means of the Biolog® Microstation System 4.2 (Biolog Inc., Hayward, CA, USA) using MT2 Microplates, designed to give the user the complete flexibility in selecting the carbon sources and in configuring the tests within the panel. The wells of the MT2 MicroPlate are “empty” in that they do not contain any pre-filled carbon sources, but each well already contains a buffered nutrient solution and the tetrazolium violet, a redox dye which colorimetrically indicates utilization of the added carbon sources. About 0.3 mg of Cervione glue or Rabbit Skin glue were added to each well ( $15 \mu\text{l}$  of  $2\% \text{ w v}^{-1}$  solutions) in order to test the ability of the bacterial strains to oxidise the target substrates. For each strain, a cell suspension was prepared in sterile phosphate buffered saline (PBS), starting from fresh BUG (Biolog Universal Growth medium; Biolog Inc.) streak plates and adjusting the cell concentration to 65% of transmittance. Before inoculation, each bacterial suspension was incubated 48 h at  $28^\circ\text{C}$  in a rotary shaker (150 rpm), in order to consume the excess of nutrients (starvation). Then the cell suspension was inoculated into the MT2 MicroPlate,  $150 \mu\text{l}$  per well. Each strain was tested in triplicate on both glues. For each strain tested, a negative control well (with no carbon

source) and a positive control well (with 0.3 mg glucose) were included. The plates were incubated at  $28^\circ\text{C}$  in the dark and analysed by the Microplate Reader (dual wavelength data:  $\text{OD}_{590} - \text{OD}_{750}$ ) after 1, 2, 3, 4, 5 and 7 days of incubation.

The strains able to oxidise one or both glues within 48 h, were tested for the growth capacity on the same substrates supplied as sole carbon source. Cell suspensions were prepared in sterile PBS at a transmittance of 65% and starved at  $28^\circ\text{C}$  in a rotary shaker (150 rpm) for 48 h. Suspensions were then inoculated (about  $10^5 \text{ CFU mL}^{-1}$ ) in 50 mL of sterile M9 mineral medium (Maniatis et al., 1982) and the two substrates were separately added at the final concentration of  $0.2\% \text{ w v}^{-1}$ . Cultures were incubated at  $28^\circ\text{C}$  in a rotary shaker (150 rpm) up to 7 days. Growth was evaluated by the plate count method, plating on TSA (Tryptic Soy Agar; Liofilchem, Teramo, Italy) medium. Positive and negative controls were prepared inoculating the bacteria in the M9 mineral medium with glucose  $0.2\% \text{ w v}^{-1}$  as sole carbon source and without any carbon source, respectively.

## 2.4. Cellulolytic activity

The bacterial strains able to grow on animal glues were tested for cellulolytic activity according to Hankin and Anagnostakis (1977), growing the strains on M9 solid medium containing  $0.5\% (\text{w v}^{-1})$  CarboxyMethylCellulose (CMC). After incubation at  $28^\circ\text{C}$  for 5 days, CMC hydrolysis was highlighted according to Sirisena and Manamendra (1995). The strains *Cellulosimicrobium cellulans* TPBF10<sup>E</sup> and *Cellulomonas* sp. TBF11<sup>E</sup> belonging to taxonomic genera known as hydrolysing cellulose polymer were used as reference strains for positive control.

## 2.5. Preparation and application of the bacterial suspension on paper specimens

Following the screening, the strain with the best prerequisite for glue degradation and no cellulolytic activity was chosen for the application on paper specimens. A bacterial suspension was obtained by inoculating 0.5 mL of an overnight culture into 50 mL TSB (Tryptic Soy Broth, Liofilchem, Teramo, Italy) then incubated at  $28^\circ\text{C}$  and 120 rpm for 48 h. The cells were centrifuged at 3000 g for 15 min, washed and suspended in PBS. Cell suspension was starved for 48 h at  $28^\circ\text{C}$  and 120 rpm. The starved suspension was centrifuged at 3000 g for 15 min and the bacterial pellet was

**Table 1**

Bacterial strains screened for selecting effective animal glues degraders, showing the time-course (hours) to reach the stationary phase of oxidation (ox) and assimilation (ass) of animal glues.

Strain	Phylogenetic affiliation	GenBank accession	Rabbit skin glue		Cervione glue	
			ox	ass	ox	ass
CONC18 <sup>E</sup>	<i>Achromobacter xylosoxidans</i>	EU275351	18 h	24 h	—	—
LAM21 <sup>E</sup>	<i>Acinetobacter calcoaceticus</i>	EU118781	18 h	24 h	18 h	24 h
LAM22 <sup>E</sup>	<i>Arthrobacter</i> sp.	EU019987	—	—	—	—
CONC14 <sup>E</sup>	<i>Comamonas</i> sp.	EU275355	—	—	—	—
DAN5 <sup>E</sup>	<i>Microbacterium esteraromaticum</i>	EU249580	—	—	18 h	48 h
TSC8 <sup>E</sup>	<i>Microbacterium oxydans</i>	EU249583	—	—	—	—
TNS15 <sup>E</sup>	<i>Ochrobactrum</i> sp.	EU249585	18 h	48 h	18 h	24 h
TPID2 <sup>E</sup>	<i>Pseudomonas alcaligenes</i>	EU249591	—	—	—	—
LAM33 <sup>E</sup>	<i>Pseudomonas fluorescens</i>	EU019991	—	—	—	—
LAM1 <sup>E</sup>	<i>Pseudomonas jessenii</i>	EU019982	—	—	—	—
AGL2 <sup>E</sup>	<i>Pseudomonas lutea</i>	EU118771	—	—	—	—
AGL13 <sup>E</sup>	<i>Pseudomonas putida</i>	EU118779	—	—	—	—
LAM 9 <sup>E</sup>	<i>Pseudomonas resinovorans</i>	EU019983	—	—	—	—
CONC11 <sup>E</sup>	<i>Pseudomonas stutzeri</i>	EU275358	18 h	24 h	—	—
CONC17 <sup>E</sup>	<i>Pseudomonas stutzeri</i>	EU275359	—	—	—	—
TNS12 <sup>E</sup>	<i>Rhodococcus erythropolis</i>	EU249592	—	—	—	—
TPID9 <sup>E</sup>	<i>Stenotrophomonas maltophilia</i>	EU263112	48 h	48 h	24 h	48 h



suspended in 50 mL of saline solution; the final cell concentration, determined by the plate count method on TSA, was about  $10^9$  CFU mL<sup>-1</sup>. The bacterial suspension was entrapped in 1% (w v<sup>-1</sup> in sterile distilled water) agar gel. Each paper specimen was covered with the gel and kept at room temperature. As negative control, a bacteria-free agar gel was applied to some specimens. After 4 and 18 h of treatment, the agar gel was removed by sterile tweezers and the swollen glue was gently removed by means of a dry sterile swab. In order to remove contingent residual bacterial cells, three final “washing” steps with a 1% (w v<sup>-1</sup> in sterile distilled water) agar gel were performed. At the end, presence of viable bacterial cells was monitored by the plate count method, by plating on TSA medium (Tryptic Soy Agar, Liofilchem, Teramo, Italy) a few samples, collected by a sterile swab from the surface of the treated specimens, and incubating at 28 °C for 7 days.

## 2.6. Scanning electron microscope analysis

SEM analyses of paper specimens before and after the treatment (about 10 for each treatment) were performed with an EVO 50 Scanning Electron Microscope (Carl-Zeiss Electron Microscopy Group; Oxford, UK). Paper fragments measuring 5–10 mm in diameter were cut and mounted on to a 12-mm metal stub (Agar Scientific, Essex, England), using double-sided carbon adhesive tape (Agar Scientific, Essex, England). Samples were observed with SEM in variable pressure mode, at 20 keV, some of the samples covered with gold with a Baltec Sputter Coater for a further analysis in high vacuum mode. The sputtering was performed under an argon gas flow, at 50 mm working distance with 0.05 mbar of pressure and a current of 40 mA, for 60 s to obtain a film of gold of about 15 nm.

## 2.7. Colorimetric measurements

Each specimen was analysed before and after the treatment. Reflectance and chromatic coordinates were measured using a spectrophotometer Techkon (mod. SP 820), TECHKON GmbH, Frankfurt, Germany). The chromatic coordinates were registered using CIE L\*(luminous reflectance), a\*(red-green), b\* (yellow-blue) coordinate system. All the values are the mean of 3 determinations on the same spot, and more than three areas were measured on the same specimen. The parameter  $\Delta E^*$  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). Statistical significance was assessed by Student's t test.

## 3. Results

### 3.1. Selection of glue-degrader strains

An early indication on the capacity of the strains to react with animal glue was provided by the oxidative activity in BIOLOGTM MT-Microplates. The resulting six positive strains, described in Table 1, were then subjected to a further screening to test their capacity to grow on glues, supplied separately as a sole carbon source. The strains *Ochrobactrum* sp. TNS15<sup>E</sup>, *Acinetobacter calcoaceticus* LAM21<sup>E</sup> and *Stenotrophomonas maltophilia* TIPD9<sup>E</sup> were able to assimilate both the glues. *Achromobacter xylosoxydans* CONC18<sup>E</sup> and *Pseudomonas stutzeri* CONC11<sup>E</sup> assimilated only the Rabbit Skin glue, while *Microbacterium esteraromaticum* DAN5<sup>E</sup> only the Cervine glue. None of the glue-degrader strains showed cellulolytic activity. *Ochrobactrum* sp. TNS15<sup>E</sup> was finally chosen for the bio-removal trials, showing high affinity for both the glues, as proved by the growth kinetics (Fig. 2).

### 3.2. Bio-removal of animal glue

Before the treatment, paper specimens appeared strongly yellowed all over the surface (Fig. 3 upper part) due to ageing. Moreover a compact layer of animal glue, used for the lining of the prints before mounting, was evident along the edges of the sheet. The treatment with living cells of *Ochrobactrum* sp. TNS15<sup>E</sup> entrapped in a 1% agar gel was effective, as shown in Fig. 3 (lower part). After 4 h of contact, the surface of all the specimens underwent a whitening and showed a reduction of the original yellowing of the paper. This effect appeared to be more evident in the specimens treated with bacterial cells, although the phenomenon can be observed to a lesser extent also in the samples treated with agar gel only.

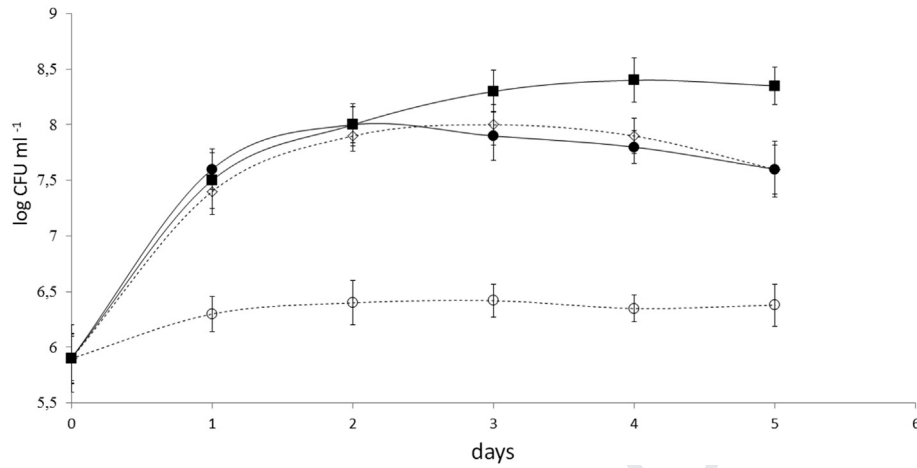
Variations of the chromatic coordinates L\*a\*b\* values after 4 and 18 h treatment are reported in Table 2. Positive  $\Delta E^*$  values occurred over time (after 4 h and 18 h treatment) where the main contribution derives from the b\* parameter, whose decrease, due to the colour's shift from the yellow to the blue direction, indicates that the original yellowing of the specimens was reduced by the treatment. Moreover, the positive variation of L\* coordinate indicates an increase of the sample brightness. Although variations of chromatic coordinates values occurred both for the specimens treated with the bacterial pack and for the controls, the application of the microbial cells significantly improved the whitening effect (Table 2).

SEM analysis confirmed these results, providing the definitive evidence of the efficacy of the treatment. The image in Fig. 4 shows the specimen partly covered by glue: it can be observed the boundary line where the glue coating ends and the cellulose fibres are evident. Before the treatment, the glue layer on the paper surface was compact, apparently dried, given the presence of cracks along the whole coating; glue agglomerates circular in shape were also evident; only a few cellulose fibres sporadically emerge from the glue layer (Fig. 5a). The treatment with *Ochrobactrum* sp. TNS15<sup>E</sup> removed the glue layer, disclosing the cellulose fibres to the surface after 4 h of contact (Fig. 5b). The effectiveness of the 18 h treatment did not differ significantly from the one lasting 4 h (Fig. 5c). The treatment with agar only was definitely not effective in removing the glue layer. The only consequences observed were the disappearance of cracks and, in few areas, the weakening of the layer evidenced by the partially visible segments of fibres (Fig. 5d). At the end of the treatment, after the final agar “washing” step, SEM analysis confirmed the absence of both gel and cells residues (Supplementary Material, Fig. S1). No viable cells were detected by the plate count.

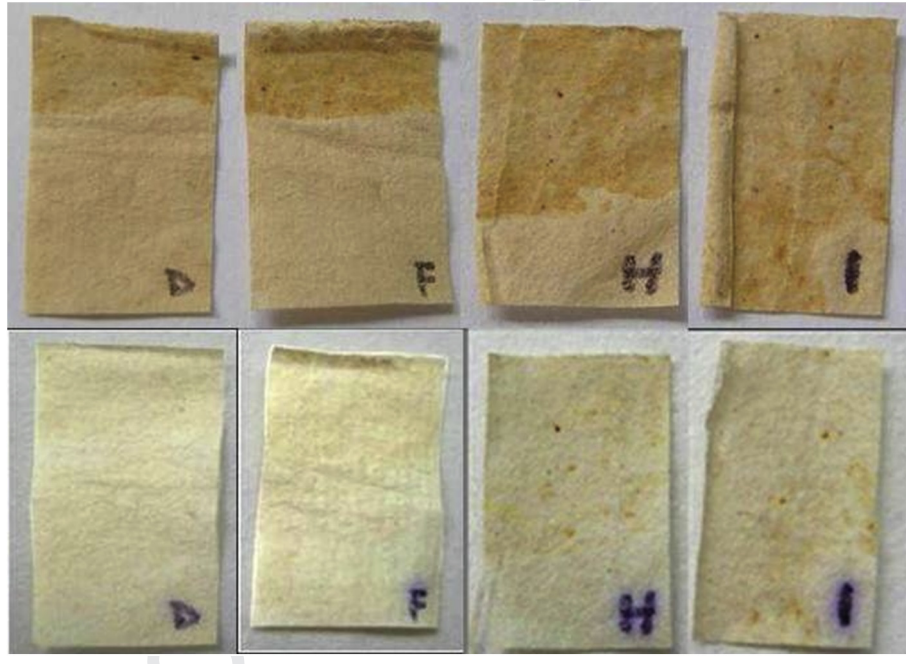
## 4. Discussion

The use of biological systems, living organisms, or derivatives thereof, to make or modify products or processes for improving the conditions of deteriorated artworks, is developing recently. Restoration procedures based on biotechnology, that goes under the name of bioremediation, is at present gaining wide acceptance and is urged by the restorers themselves, due to a growing need to move towards safe, compatible and low-cost products, according to the key principles of a more sustainable conservation strategy. Beside this reason, bioremediation can assist in the resolution of unanswered problems, including the removal of animal glues from paper material, to which this research is addressed.

Compared to purified enzymes technique, the living cells application seems to be less sophisticated and more efficient, due to the major reason that living cells show a huge potentiality to induce/speed up a chemical reaction (up to 106 times) and act like an enzymes factory, producing both constitutive and inducible enzymes, according to the needs dictated by environmental



**Fig. 2.** Growth curves of *Ochrobactrum* sp. TNS15<sup>E</sup> on Rabbit skin glue (full square) and Cervione glue (full circle), compared with the positive (glucose; open diamond, dotted line) and negative (no carbon source; open circle, dotted line) controls.



**Fig. 3.** Paper specimens before (upper part) and after (lower part) the treatment. The samples signed with letters D and F were treated with the strain *Ochrobactrum* sp. TNS15<sup>E</sup> immobilised in agar gel for 4 h and 18 h, respectively; the specimens signed with H and I were treated with the agar gel without bacteria for 4 h and 18 h, respectively.

**Table 2**

Variations of the chromatic coordinates of the paper specimens treated with bacterial cells immobilised in agar gel (TNS15<sup>E</sup>) and with a bacteria-free agar gel (control).

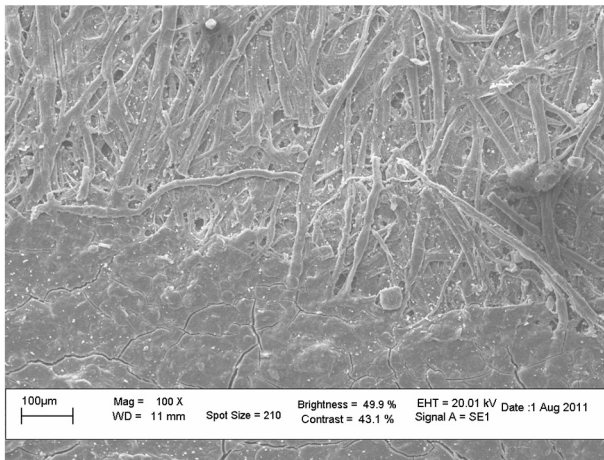
		$\Delta L^*$	$\Delta a^*$	$\Delta b^*$	$\Delta E^*$
TNS15 <sup>E</sup>	4 h	6.8 ± 0.68	−2.5 ± 0.28	−14.6 ± 0.16	16.3 (a)
	18 h	6.1 ± 0.41	−3.1 ± 0.05	−17.6 ± 0.92	18.9 (b)
Control	4 h	4.1 ± 0.8	−2.4 ± 0.13	−9.7 ± 1.7	10.1
	18 h	3.1 ± 0.5	−1.8 ± 0.12	−11 ± 1.2	11.5

Student's t test: (a)  $p < 0.05$ ; (b)  $p < 0.01$ .

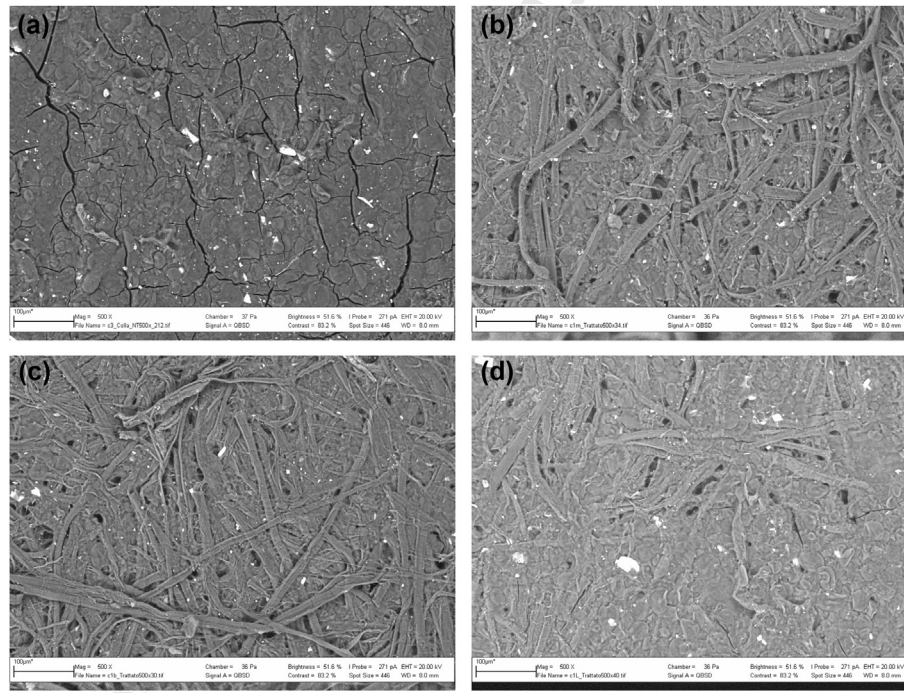
circumstances. In addition, living cells maintain homeostatic processes including iron and metal homeostasis, pH homeostasis and membrane lipid homeostasis that guarantee the survival of bacteria, allowing the ability to quickly adapt to change (Hutkins and Nannen, 1993; Andrews et al., 2003; Zhang and Rock, 2008; Krulwich et al., 2011).

The main precautions to keep in consideration regarding the application of viable bacterial cells consist in the use of safe procedures for the operators, the manufacture and the environment, by using non-pathogen microorganisms, selective towards the substrates to be removed, powerful enough to obtain a correct and desired degree of removal of the deposits, without leaving any





**Fig. 4.** SEM image of an untreated paper specimen showing the boundary line between the glue coating (bottom) and the cellulose fibres (top) [100x; scale-bar 100 µm].



**Fig. 5.** SEM images of paper specimens: particular before the treatment (a), after 4 h (b) and 18 h (c) treatment with *Ochrobactrum* sp. TNS15<sup>E</sup> immobilised in agar gel; after 18 h treatment with agar gel free of bacteria (d) [500x; scale-bar 100 µm].

residual biological activity.

To this purpose, a number of microbial strains belonging to the ENEA-Lilith collection were tested to select those able to degrade animal glues while satisfying the above requirements. These criteria led to the exclusion of spore-forming microorganisms to avoid the presence of quiescent life forms, which could germinate even in the future.

The selected bacteria are environmental strains, previously isolated from different environments, including works of art. They generally grow between 5 °C and 28 °C. For application indoor, where the temperature fluctuations ranges between 18–22 °C, this would avoid submitting the artwork to conditions very different from those in which it is generally conserved, preserving at the same time the balances that have developed over time between the

object and the conservative environment. Moreover, this feature is advantageous, even in outdoor applications, as we had evidence that low temperatures did not adversely affect the biological activity of living cells during the biocleaning of mural paintings (Mazzoni et al., 2014) carried out in winter season (about 6 °C).

Following the results of the screening, the strains *A. calcoaceticus* LAM21<sup>E</sup>, *Stenotrophomonas maltophilia* TIPD9<sup>E</sup> and *Ochrobactrum* sp. TNS15<sup>E</sup> were able to grow on both Cervione and Rabbit Skin glue, up to three orders of magnitude higher than the inoculum. Among these, *Ochrobactrum* sp. TNS15<sup>E</sup>, isolated from the internal walls of an Etruscan tomb (Sprocati et al., 2008) was selected for the application. Beside its good performance in growing on glues (Fig. 2), the genus *Ochrobactrum* had never been described as a glue degrader nor yet exploited in any bioremediation application, while it was reported as a bioremediation agent against polycyclic aromatic hydrocarbons and heavy metals (Cheng et al., 2010; Arulazhagan and Vasudevan, 2011).

The experiences described in literature on glue removal from manmade artistic works by means of living bacterial cells relate so

far to the use of a single strain, *P. stutzeri* A29, applied for the reclamation of mural paintings (Ranalli et al., 2005) which had been removed from the walls using the “tear-off” technique, by covering the surface with a strong cloth bound with generous layers of formaldehyde-treated glue (Antonioli et al., 2005).

In the case of paper material, the vulnerability of the support required much care in developing a gentle procedure. For this reason, the biocleaning treatment was performed through the use of living cells of *Ochrobactrum* sp. immobilised in an agar gel as a delivery system. Immobilisation represents a stable, consistent and protective microenvironment for microorganisms, assisting the operators in overcoming the difficulty to handle enzymes and cells that may be unstable in the unconventional conditions that occur during the operations of restoration.



Compared with liquid suspension system, the immobilisation technology offers some advantages, such as a higher biomass, improved functional properties, intensified metabolic activity and strong resistance to toxic chemicals (Cassidy et al., 1996; Liu et al., 2012).

Immobilization, simplicity and stabilization have to be strongly related concepts (Guisan, 2006). Over the last 30 years, a number of protocols for the immobilization of cells and enzymes have been reported in scientific literature, mainly devoted to biofuels and treatment of toxic pollutants (Martins et al., 2013). The use of agar as delivery system for biocleaning of cultural heritage stone surfaces and frescoes was described in Bosch-Roig et al. (2013). Cleaning artworks with gels was widespread during the last decades (Carretti et al., 2010). Aqueous, non-aqueous and mixed gels have been devised to remove stains from stone (Wheeler, 2007), varnishes and overpaint from paintings (Wolbers, 2000) and adhesives from paper (Warda et al., 2007). In particular, in the case of materials that are highly susceptible to contact with aqueous solutions, such as archival materials, the use of gel for entrapping liquid solvents in its pores matrix minimises the detrimental effects resulting from the contact of liquid solutions with the surface of the material to be treated. The slow release of solvent across the gel interface reduces the risk of mechanical stresses on paper and of swelling of paint layers, if present. In case of biocleaning, the microbial cells colonise the gel allowing a close contact with the surface to be treated.

Different concentrations of agar were tested to select the optimum concentration that would not release residues of agar after the treatment. A 1% agar has been finally selected allowing the right swelling of the glue without the release of agar residues. The application of the agar gel containing cells of *Ochrobactrum* sp. TNS15<sup>E</sup> produced an effective glue removal after 4 h of contact at room temperature, allowing the complete removal of the compact layer of glue from the surface of the paper specimen.

An immediately visible result of the application is a general whitening of the samples due to the elimination of the glue used in the print's mounting and also to the removal of the initial yellowing of the specimens caused by the natural ageing of the paper. This whitening phenomenon is visible also on the controls, treated with a bacteria-free agar gel, but the presence of microbial cells definitely enhances this effect (Fig. 3). The analytical evaluation of the treatment efficacy was performed by means of colorimetric analyses, in order to measure the aesthetic effects of the application and by means of SEM observations in order to prove the removal of the glue coating. The colorimetric measurements allowed to assess the whitening of the specimens (Table 2); an increase of the L\* coordinate, which expresses lightness, was noticed, a sign that the reflectance of the samples increased. Moreover, the  $\Delta E^*$  had a considerable change, increasing significantly enough to justify the perception of the colour variation after treatment also by the naked eye. The application of the microbial cells greatly improved, compared to control, the whitening effect, as shown by the colorimetric coordinate although some variation occurred also in the control.

SEM analysis allowed to evaluate the extent of the glue removal and to verify if gel residues were left after the final "washing" steps. SEM analysis showed evidence of the removal of the adhesive. Cellulose fibres, previously hidden by the thick layer of glue were disclosed after 4 h of contact with the bacterial pack. The control, conversely, treated with a bacteria-free agar gel, showed the permanence of a glue layer and significant glue residues, characterized by globular clusters, visible on the surface of the examined area. The effectiveness of a 18 h-treatment does not differ significantly from that lasting 4 h, therefore we believe the contact time may be optimized with a slight deviation from 4 h. The possibility

to apply for a shorter time the bacterial pack is of great importance in this cleaning procedure, because of the fragile nature of ancient paper.

A final, accurate cleaning phase is a very important aspect in biocleaning. Indeed, at the end of the treatment all residues of both cells and cell-carrier support used should be removed carefully to avoid both the continuation of undesired metabolic processes and possible secondary colonisation due to the remains of the organic gel material. Thus, an adequate strategy for artwork protection must be planned; if the microorganisms remain alive and active they can cause material loss or damage, either directly, or as a consequence of their catabolic by-products. At the same time all residual gels and bacterial cells, even if not alive, must be eliminated because they can represent a nutritive source for other heterotrophic microorganisms that could be responsible of a secondary colonisation with further damage to the artwork. For this reason, the final "washing" after the treatment was carried out with three successive packs of a gel agar in distilled water, ensuring to leave the surface free of any organic residues.

## 5. Conclusions

The procedure deriving from the study presented in this paper demonstrated the feasibility of cleaning ancient paper from aged animal glue through the use of whole living microbial cells.

This achievement marks a progress beyond the state of the art showing advantages over current methods mostly based on aggressive mechanical and chemical approach, or on the challenging method with purified enzymes.

This simple and gentle procedure overcomes a series of drawbacks of those methods. It is selective, it safeguards the integrity of paper because it is non-aggressive, it does not produce holes and tears, it avoids excessive hydration of the paper, it does not weaken cellulose, it is low-cost and toxicity-free. Moreover, neither skilled operators nor optimal application conditions are required.

A further novelty lies in the use of living bacterial cells to selectively remove organic deposits from an original organic substrate.

Demonstrating the feasibility of this procedure, this work contributes to widen the spectrum of applicability of biotechnology in this field and hints at a promising expansion of green and innovative techniques of restoration for even the most vulnerable materials, such as ancient paper.

It is needed, at last, to convert the microbial potential into readily-available and easy-to-use products for the restorers, to create a bio-based market suitable for the solution of a diverse set of restoration problems.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ibiod.2015.12.019>.

## Uncited reference

Tiano et al., 1999.

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