Pilar Bosch-Roig

Biocleaning of animal glue on wall paintings by Pseudomonas stutzeri

PILAR BOSCH-ROIG[1]*, JOSE L REGIDOR-ROS[1], PILAR SORIANO-SANCHO[1], ROSA M MONTES-ESTELLÉS [2]
*Corresponding author
1. IRP, Universitat Politècnica de València Camino de vera, s/n, 46022 Valencia, Spain
2. Universitat Politècnica de València, Biotechnology Department, Camino de vera s/n, 46022 Valencia, Spain

KEYWORDS

Biocleaning, Pseudomonas stutzeri, mural painting, Santos Juanes Church, animal glue.

ABSTRACT

The article focuses on the Biocleaning of indoor wall paintings subjected to animal glue alterations applied in old restorations. The cleaning difficulties by traditional restoration methods make biocleaning to be a remarkable alternative. Biocleaning is a cleaning strategy that uses a viable non-toxic bacteria strategy. Presented in this article is the research about the Pseudomonas stutzeri cleaning of animal glue from the eighteen-century wall paintings of the Santos Juanes church of Valencia, Spain.

INTRODUCTION

Cultural Heritage surfaces can be subjected to damage due to wrong restorations. Organic substances applied by earlier restoration treatments can produce important pathologies in the artworks - like darkening, yellowing, dirt retention, desquamation, permeability modification - which can greatly modify the fresco aesthetics and even lose pictorial fragments. These substances can include natural compounds such as animal, vegetable glues, egg casein, walnut etc.
The idea of utilizing bacterial cells for the restoration phase of works of art, evolved due to the fact that the majority of the microorganisms have a positive role, with a minority of these causing biodeterioration. This positive use of microorganisms for the clean of cultural heritage is called: Biocleaning.
Biocleaning shows advantages over traditional restoration methods, mainly when the substances to be cleaned are difficult to remove. In these cases, chemical restoration methods are not specific and can be aggressive to the artwork. To degrade complex molecules, a mixture of enzymes is required. This mixture, however, is sometimes not available commercially or cannot be added simultaneously due external specifications (application times, temperatures, etc). Conversely, bacteria are able to synthesize precise enzymes for the substances in need of removal (1).
Biocleaning of the unwanted organic substances present on wall paintings has only been successfully carried out in the Camposanto Frescoes of Pisa, Italy (2). Two large frescoe painted in the 14th Century - the first by Spinello Aretino and Andrea Bonaiuti (3.5 m x 7.8 m), and the second by Buonamico Buffalmacco (6.10m x 15.65 m) - were satisfactory cleaned with bacteria. Both wall paintings had an altered animal glue layer covering them and residual traces of casein which were difficult to remove by traditional restoration treatments. The animal glue was present as a consequence of past restorations that aimed to have *strappo* removed from the wall. Viable cells of *Pseudomonas stutzeri* A29 strain (from 2 to 12 hours treatment) were used in combination with proteolytic enzymes (Protease Type XIX from *Aspergillus sojae* and Collagenase Type IA and Type V from *Clostridium histolyticum*) to remove 95% of the animal glue (3, 4). The efficacy of the biocleaning was detected using pyrolysis. Microbial monitoring shows that *P. stutzeri* cells were not present in the wall painting after the treatment and no negative effect due to the metabolism was found on middle and long term controls.
Many other biocleaning applications confirm the effectiveness of this methodology. Sulphur-reducing bacteria and nitrate-reducing bacteria have been used for cleaning black rust from stone materials (5, 6). For example black crusts on the ornamental fragments of a lunette in the Cathedral of Milan were cleaned applying *Desulfovibrio vulgaris* (7, 8). Chalk residue on the Pietà Rondanini sculpture by Michelangelo was removed using *Desulfovibrio vulgaris.*
Nitrates and sulphates were cleaned in the external walls of the Matera Cathedral by using nitrate-removing bacteria *Pseudomonas pseudoalcaligenes* or *Paracoccus denitrificans* (9, 10, 11).
Our multidisciplinary research group has previously shown the efficient bioremoval of insoluble salt efflorescence of frescoes present in the lunettes of the Santos Juanes Church, in Valencia, Spain. Viable *Pseudomona stutzeri* cells were used to clean, during a 2h treatment, the 84% of the nitrates present on the frescoes (12).
This article shows the biocleaning of insoluble animal glue present at the frescoes of the central vault of the Santos Juanes Church, in Valencia, Spain.
The Santos Juanes is a Baroque church situated in the main old area of Valencia, Spain, and it's considered to be one of the most important buildings of the city. The central vault and lunettes of the church are covered by around 1200m^2 of wall paintings; all of them are dated between 1693 and 1702 and were painted by Antonio Palomino and Guilló (see Figure 1).
In 1936, during the Spanish Civil War, several deliberated fires were set inside the church and the wall paintings were highly damaged. In the 60's, an incorrect restoration was carried out detaching the frescoes from the wall using the *strappo* technique (13).

This inadequate restoration produced many different problems to the frescoes, being one of them the incorrect removal of animal glue used during the *strappo*. Any attempts to remove this animal glue using traditional techniques have been unsuccessful. The research presented in this article shows an alternative method, based on the use of viable bacteria, to clean old and encrusted animal glue on wall paintings.

Figure 1. Image of the wall paintings on the central vault of the Santos Juanes Church in Valencia, Spain.

EXPERIMENTAL SECTION

First of all, UV light pictures (Nikon D90) and scalpel micro-samples were taken from the fresco surface before the biocleaning treatment. The micro-samples were analysed in the laboratory for Infrared Spectroscopy Fourier Transform (FTIR), optical microscopy (Leica DMR microscope and a stereoscopic microscope Leica GZ6), Pyrolisis and Gas-Mass Chromatography.
These analyses were performed in order to characterize the organic nature of the matter.
Once the nature of the organic matter to be cleaned had been characterized, the adequate bacteria were selected in the laboratory. *Pseudomonas stutzeri* was selected to be used to remove the animal glue from the frescoes since it had already been succesfully used for the same type of biocleaning in Pisa in 2005. Diverse *P. stutzeri* strains were assayed in the laboratory being strain 5190 the selected
This strain was choose because previous works (14) showed *Pseudomonas stutzeri* 5190 to be one of the most effective on biocleaning of animal glue with high Protease activity. The selected bacteria were bought in the German collection of bacteria type (DSMZ) and optically analysed in the laboratory by Cryo scanning electron microscopy (JEOL JSM5410 with a cryo-preparation Oxford Instruments CT1500C system).
Exponentially growing bacterial suspensions containing about 10^9 ml $^{-1}$ viable cell were needed for the biocleaning treatment. They were obtained by inoculating 100ml of an overnight animal glue-culture broth into a 1L animal glue broth flask (M9 mineral medium, supplemented with 1% of animal glue) which was incubated at 28°C in a shaker (200 rpm). Afterwards a 24h centrifugation (3x 4200rpm for 10m) was carried out and the pellet was washed twice with NaCl 0.8% pH 7.0 and re-suspended in sterile water. The aqueous cell suspension was immediately transported to the church on ice or stored at 4°C for a few days.
The application procedure was carried out following four steps: 1st Direct application of *P. stutzeri* in an aqueous suspension on the fresco surface with a brush (Japanese paper can be added as a protective if the painting layer is fragile); 2nd A humid carrier (2% concentration, 5mm deep thin layer of European bacteriological agar) is added and the surface is maintained in constant temperature (22°C-30°C) by using infrared heat lamps; 3rd After 2h of treatment the agar is removed with a spatula and the surface is cleaned with sterile water; 4th Finally the treated area is dried at room temperature.
Before using biological cleaning methods on the fresco, laboratory analysis on sample tests were carried out. Stone material simulating wall paintings with a layer of animal glue were used to do the laboratory analysis.
The laboratory tests determined the number of bacteria needed, the treatment's length, the adequate temperature, etc. by applying to the sample tests different parameter conditions. Once all the different parameters were fixed, it began the process to clean the old and insoluble animal glue present on the wall paintings of the Santos Juanes Church.
Two types of control tests have been done: a) wall painting area not treated; and b) wall painting area treated with agar but without bacteria.
The efficacy of the cleaned treatment was controlled by visible and UV-light pictures, by FTIR analysis and by Gas-Mass Chromatography in order to detect the organic matter removal with the biocleaning treatment. Water Absorption index was also determined (using a contact sponge kit, CTS-Spain) before and after treatment to detect the correct animal glue removal directly related with an increase in the fresco water absorption index (15).
A control of the microbial absence on the treated surface and viability is always carried out with contact plates- colony counts and total ATP assays (using a 3M™Clean-Trace™ NG Luminometer).
Statistical analyses of variance (ANOVA) were executed to evaluate differences between the control areas (cleaned with agar and water) and the biocleaned areas (cleaned with agar and bacteria), and to evaluate the differences before and after the biocleaning. These results are shown by P values< 0.05.

RESULTS AND DISCUSSION

The analytical characterization (by Pyrolysis and Gas-mass Chromatography) of the organic matter found on the detached fresco of the Santos Juanes Church determines that the main component was animal glue (Figure 2). The type and relation of amino acids presents in the organic matter of the fresco and in particular the presence of hydroxiproline allow us to identify it because it's an amino acid characteristic of the gelatine.
In order to proceed with a correct biocleaning process, an adequate carrier is needed. Previous biocleaning studies have used carriers made with inorganic materials like sepiolite (16), or organic materials like carbogel, cotton (17) or agar (18).

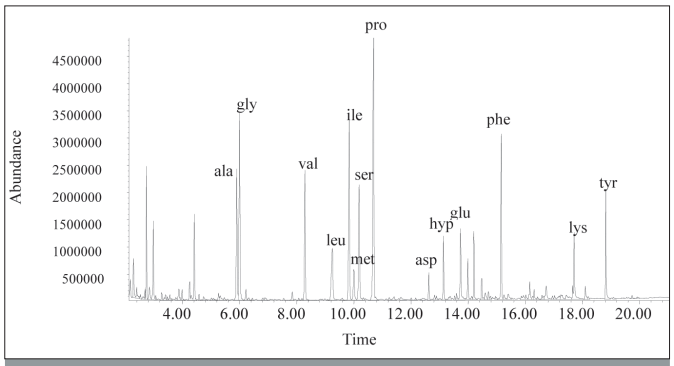

Figure 2. GC-MS chromatography of a sample taken from the fresco's surface. Alanine (ala), glycine (gly), valine (val), leucine (leu), isoleucine (ile), methionine (met), serine (ser), proline (pro), aspartic acid (asp), hydroxiproline (hyp), glutamic acid (glu), phenilalamine (phe), lysine (lys) and tyrosine (tyr) can be identified.

The carrier must be able to supply water to the bacteria during the duration of the treatment without interfering with the art-work. In this case we decided to use an agar carrier. Agar showed to be the most appropriate carrier, both in the sample laboratory tests and in the on-site experiments. It has good adhesive properties when applied onto vertical and oblique surfaces; it has good bacterial retention in its surface providing them adequate water without interfering with the wall surface and it is able to slowly release water only in the surface which allows homogeneous cleaning.

10^9 ml^{-1} viable cell of *Pseudomonas stutzeri* was applied in an aqueous solution and with a brush directly onto the fresco surface. Agar was added as a carrier and the biocleaning treatment lasted 2 hours. After those 2 hours, the agar was removed and the surface was cleaned using sterile water and dried. The results were evident at simple sight (Figure 3), however, in order to confirm them, diverse tests were carried out to determine if the animal glue had been completely removed. Analytical Pyrolysis and Gas-Mass Chromatography tests showed the animal glue had disappeared. A semi-quantitative measurement of the animal glue remove was done by measuring the IR absorption bands (2900 cm^{-1} and 2800cm^{-1}) of the organic components obtained by FTIR analysis.

These areas were divided by the calcic carbonate IR absorption band (1935cm^{-1}) area, chosen because is the principal component of the fresco.

The quotient obtained in the fresco before the cleaning was 0.02, after the biocleaning was 0.01 and after the control cleaning (with agar and water) was 0.016. These results showed how the bacteria are able to produce a higher reduction of the organic matter present on the fresco compared with water control treatments.

The study of the water absorption (Wa) index before and after the biocleaning treatment showed statistically significant differences (p-value= 0.0012). The average of Wa index before the cleaning was of 0.008 and the Wa index after the biocleaning was 0.017 and the Wa index in the control areas cleaned only with agar (without bacteria) was 0.003 before the cleaning and 0.004 after the cleaning, not showing statistically significant differences (p-value=0.65).

The differences on the Wa of the wall painting, which had doubled after the biocleaning proves that the animal glue layer present on the fresco's surface had been successfully removed. The elimination of this layer allowed water to easily enter inside the wall painting, increasing therefore the Wa index. UV-light analysis before cleaning showed a big quantity of diverse sediments covering the pictorial surface, as can be seen in the yellowing and bluing tones of Figure 4.

Figure 3. View of the particular wall painting area before (a), during treatment with agar as a carrier (b), and after (c) bacterial treatment. Animal glue rests can be seen in the left image (a) as black spots, and their correct removal in the right image (c).

However it was impossible to detect significant differences on the UV-light analysis after the biocleaning. This is due to the fact that the distribution of the glue is very irregular and not strictly superficial and also due to the presence of resinous repainting and exudation of calcium casein that gives a high distorted UV picture. Therefore the visible light examination, the water absorption index and the chemical analysis indicate that the biocleaning was developed correctly.

Finally, a microbiological control of the 600cm^2cleaned surface was carried out to ensure that no alive bacteria had been left on the fresco surface and in order to avoid the risk of microbial growth on it. Just after the treatment has finished, fast ATP content analysis (by luminescence detection) and contact plates sample were taken; the ATP is an energetic molecule common to all organisms that can be used as a bio-indicator of the levels of microbial activity (19). On the other hand contact plates, after 48 hours of incubation; reveal information about the presence or absence of bacteria in the treated area. Very low ATP values (144 URL) and little colony forming units on the contact plates (6 CFU) were found on the treated area.

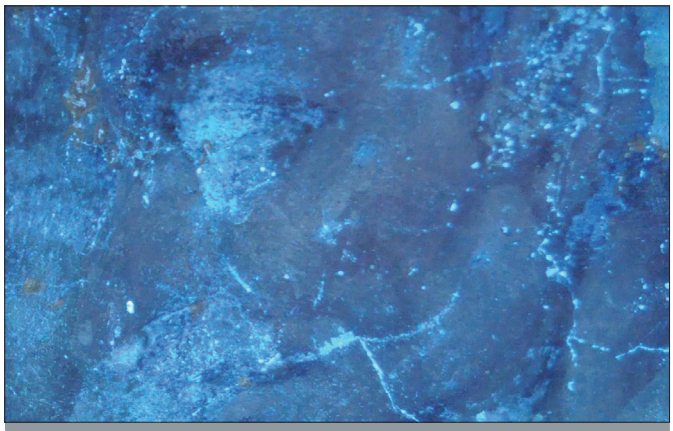

Figure 4. Fresco fragment UV light picture before the biocleaning treatment.

At the same time no significant differences were found between treated and not treated areas (100URL and 2CFU). These results show that this kind of biocleaning procedure is safe and risk-free when treating wall paintings.
Analyses have been carried out to determine the bacterial solution quantity needed to bioclean animal glue present on wall paintings. Our analyses conclude that an average of 0,2L of bacterial solution is needed to clean one square meter of wall paintings, using agar as a carrier. Previous studies showed that 2L bacterial solution was needed to clean one square meter of wall paintings when using cotton as a carrier (20), this implies that agar reduces ten times the required bacteria what has an important economical repercussion on the technique and supports the growing evidence of the benefits of using agar instead of cotton as a biocleaning carrier.
This work proves once again that biotechnologies applied to restoration of cultural heritage are a successful approach. It presents a correct biological case of animal glue cleaning from 18th century frescoes with short term application of *P. stutzeri* DSMZ 5190 and agar.
All these results support the evidence that this technology is risk-free for the restorers since only non-pathogenic bacteria are used in the procedure. At the same time it is a non-invasive technique for the artworks and easily monitored since these bacteria are unable to produce spores, meaning that all bacteria will die if the surface of the artwork is dried out (21). On the other hand this technique is very specific due to a precise enzyme production of the bacteria (22) and it is as well an environmentally friendly cleaning due to the lack of toxicity of the technology. This treatment appears as "the unique way" known apart from the direct use of enzymes to remove old glue without any harm to the substrate as it has been demonstrated with all the tests and in situ treatments done in this field since 2005. Future test must be done to ensure this affirmation in comparison with the new technologies that are every day appearing. The low–cost of this cleaning procedure previously demonstrated in a recent work of Lustrato et al. 2012 is here also augmented. The costs of this technique are mostly comparable with the normal chemical-physical techniques but the biological technique is more opportune because it does not produce any harm to the art works. If costs are compared with the use of enzymes (which is a comparable technique in terms of efficiency and lack of art works damage) we can easily see that biocleaning is low cost, because protease cost is about 150€/L and collagenase cost is 500€/L but biocleaning cost is 90€/L as described by Lustrato et al 2012.This economic cost increases the competence of this technology which has attracted diverse private enterprises to commercialize this biotechnological approach. It is important to remark the interdiciplinarity of this research project, where microbiologists, restorers, art historians, chemists...work together.

CONCLUSION

This study shows an efficient biocleaning of animal glue rests present on the wall paintings of the Santos Juanes Church of Valencia using *P. stutzeri* DSMZ 5190 and agar. Affirming that short time applications (2h) are enough to efficiently bioclean old and incrusted animal glue rests from wall paintings, according to the Lustrato et al. 2012 new paper.
The biocleaning technological approach offer restorers a different approach for conservation of cultural heritage. This technology uses non-pathogenic and non-spore forming bacteria which is naturally present in the environment that allows adequate and effective wall painting cleaning without using traditional toxic restoration reagents.

REFERENCES

1- G. Ranalli., M. Matteini et al., Of Microbes and Art: the role of microbial communities in the degradation and protection of cultural *heritage*. Kluwer Academic/Plenum Pub. Company, 2321-245 (2000).
2- G. Ranalli, G. Alfano *et al., Journal of Applied Microbiology*, **98**, pp. 73-83 (2005).
3- P. Antonioli, G. Zapparoli, et al., *Proteomics*, **5**, pp. 2453-2459 (2005).
4- G. Lustrato, G. Alfano et al., *International Biodeterioration & Biodegradation*, **69**, pp. 51-61 (2012).
5- K. Heselmeyer, U. Fischer, et al., *BIOforum,* **1/2**, p. 89 (1991).
6- G. Ranalli, G. Lustrato, et al., *Annals of Microbiology*, **59**, p. 19 (2009).
7- F. Cappitelli, L.Toniolo, et al., *Applied Environmental Microbiology*, **73**, pp. 5671-5675 (2007).
8- Italian Patent: Procedimento di biopulitura di superfici di manufatti di diversa natura chimica ed edifici. MI2006A000776 (2006). Intellectual Property Rights: University of Milan & University of Molise.
9- F. Cappitelli, E. Zanardini, et al.,. Geophysical Research Abstract, **7**, pp. 24-29 (2005).
10- E. May, A.M. Webster et al., *The BIOBRUSH project for bioremediation of heritage stone, Heritage Microbiology and Science. Microbes, Monuments and Maritime Materials,* The Royal Society of Chemistry Publishing, 76-93, (2008)
11- A. Webster & E. May. *Trends in Biotechnology*, **24**, pp. 255–260 (2006).
12- P. Bosch-Roig, R. Montes-Estellés, et al., 15th International Biodeterioration & Biodegradation Symposium, 19-24, (2011)
13- P. Roig-Picazo, I. Bosch-Roig, *La Iglesia de los Santos Juanes de Valencia, proceso de intervención pictórica 1936-1990*, Polytechnic University of Valencia (1990).
14- G. Ranalli, G. Alfano *et al., Journal of Applied Microbiology*, **98**, pp. 73-83 (2005).
15- P. Tiano, C. Pardini, ARKOS, **5**, pp. 30-36 (2004).
16- G. Ranalli, M. Chiavarini et al., International Biodeterioration & Biodegradation, **40**, pp. 255-261, (1997).
17- P. Bosch-Roig, J.L. Regidor-Ros et al., *Arché*, **4-5**, pp. 115-122, (2010).
18- P. Bosch-Roig, J.L. Regidor-Ros et al., *International Biodeterioration & Biodegradation* (2012)
19- G. Ranalli, C. Belli et al.,. Macromolecular Symposia, **236**, pp. 98-104 (2006).
20- G.Lustrato, G.Alfano et al., *International Biodeterioration & Biodegradation*, **69**, pp. 51-61 (2012).
21- M. T. Madigan, J.M Martinko et al., *Brock Biology of Microorganisms*, Pearson International Edition,12 edition pag167 (2009).
22. P. Antonioli, G. Zapparoli et al., *Proteomics,* **5**, pp. 2453-2459 (2005).