SULFATE REDUCING BACTERIA AS BIO-CLEANING AGENTS: DEVELOPMENT OF NEW METHODOLOGIES AND STUDY CASES

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1. Introduction

Sulfation is a very common pathology in stone, especially in urban areas, due to air pollution.

Sulfur dioxide (SO_2) is a pollutant which converts to sulfite ions (SO_3^{2-}) in the presence of moisture and is then oxidized to sulfate ions (SO_4^{2-}) by oxidizing agents such as oxygen, ozone and hydrogen peroxide [1]. In these conditions, sulfates interact with the calcium carbonate of the stone forming bi-hydrate calcium sulfate or gypsum ($CaSO_4 \cdot 2H_2O$). Gypsum undergoes processes of dissolution and recrystallization on areas exposed to rain, resulting in the erosion of the altered stone. Moreover, if the surface is not exposed to rain, minerals and smog particles could become entrapped in the porous surface of the gypsum, leading to the formation of black crusts [1].

Traditional chemical and mechanical treatments are not selective towards the alteration, attacking not only the black crust but also the sound stone. An alternative cleaning technology, employing sulphate-reducing bacteria (SRB), was first proposed

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by Atlas and colleagues (1988). They used a specific strain of *Desulfovibrio* for the removal of sulfates from a stone cornice of the Museum of Natural History in Chicago by immerging the sample in a broth culture for 24 hours. The experiment was successful and the bacteria were able to dissociate the gypsum [2]. In fact, due to its metabolism, *Desulfovibrio vulgaris* reduces sulphate ions while calcium ions react with carbon dioxide allowing the precipitation of calcite, according to the following reaction:

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

From that first experiment, other tests were done until one research group at the University of Milan patented some microbial cells as cleaning agents, including *Desulfovibrio vulgaris.*

This innovative and environmental friendly cleaning technology has been applied successfully on important cultural monuments, including two artworks by the sculptor J. Eberle, used to decorate the *Castle of Buon Consiglio* of Trento [3]; G. Lazzerini's sculpture "*Allegoria della Morte*" [4]; one tile from the Cathedral of Milan [5]; and one area of the base of the *Rondanini Pietà* [6].

In light of interesting development opportunities and potential market externalities, the University spin-off Micro4yoU acquired the exclusive patent by initiating the project Micro4Art, aimed at implementing a line of biological products and diagnostic services, and training and assistance for restoration professionals. The first line product is Micro4Art-sulfates, an appropriate microbial formulation for treating stone surfaces and for effectively removing sulfate alterations [7].

Initially, the product was a prototype, it was produced in laboratory and directly applied, but with huge limitations such as: *i*) low productivity; *ii*) high production costs; *iii*) impossibility to store the product for long periods. For the removal of particularly thick and persistent black crusts, the application of the microbial formulation has to be carried out several times and though effective, affects the global cost of restoration. Lastly, although mural paintings suffer from this phenomenon of sulfation, no testing has yet been done.

2. Optimization of the productive process and bacterial biomass production

The research project, finalized to optimize the productive process of *D. vulgaris* and to develop a correct method for bacterial biomass long-time conservation, consisted of several steps.

Initially, the previous laboratory protocol was reproduced in 3 L flasks in order to establish the bacterial growth curve, verify its metabolic response at different growth conditions, and identify the parameters to act on in order to improve biomass production. By shifting the fermentation process from flasks to pilot plants (9 and 75 L fermenters), it was possible to control the main parameters, such as pH and H_{2S} concentration. Indeed, an over-accumulation of hydrogen sulfide is toxic for bacteria and causes a drastic reduction in pH, leading to unfavourable growing conditions.

In their entirety, these adjustments have increased the production of bacterial biomass (at least two orders of magnitude) and have reduced production times by about 40%.

Regarding its conservation for long periods, the biomass was lyophilized testing different cryoprotectants. The addition of lactose was the most effective in terms of post-rehydration cellular vitality among all the methods utilized, ensuring product stability for not less than 12 months [8].

3. Study cases

3.1 The statue of Neera

The development of new application methods using the microbial Micro4Art-sulfates formulation, finalized to reduce the time and number of applications needed for sulfate alteration removal, had been successfully applied on the funeral monument of the writer Anna Zuccari, pen name Neera, built at the beginning of the 1900s and currently located in the *Famedio* of the Monumental Cemetery of Milan [7].

3.1.1 Conservation state of the artwork

Before restoration, the overall state of conservation of the artifact was fair. Moreover, before the statue was moved, it was subject to the constant mechanical, physical and chemical actions of atmospheric agents, as well as the natural aging of the marble, all of which contributed to deterioration. In particular, before starting restoration, two different kinds of alteration were present: one typical of run-off zones, in which the surface of the marble appeared compact but very rough and grainy, also characterized by an extensive superficial and intergranular deposit involving widespread darkening of the surface; the other, typical of those areas protected by run-off, was characterized by black crusts with sulfates. The clear division of these areas gave rise to a marked chromatic alteration of the artwork.

3.1.2 Biocleaning assays

The “*Soprintendenza per i Beni Architettonici e Paesaggistici per le Provincie di Milano, Bergamo, Como, Lecco, Lodi, Pavia, Sondrio e Varese”* (arch. L. Corrieri, official responsible for the procedure – reference number 6732) commissioned the University spin-off Micro4yoU to perform biocleaning assays on alteration by sulfides on the statue. The method suggested by the spin-off included the use of Micro4Art-sulfates bio-formulation based on sulfate-reducing bacteria.

The Micro4Art bio-formulation was applied both alone or with a non-ionic surfactant, commercially known as Tween20. Tween20 is commonly used in restoration and cleaning procedures on stone surfaces with a concentration range between 3%-10%. Being a very gentle aqueous detergent, Tween20 guarantees high compatibility with microbial cells and hence it can be used with the Micro4Art bio-formulation. The treatments on small tested areas of the statue were done as follows:

Treatment 1: 7 applications of 22 h each with Bio-treatment (Micro4Art)
Treatment 2: 3 applications of 22 h each with Surfactant (Tween20)
Treatment 3: 1 application of 22 h with Surfactant + 2 applications of 22 h with Bio-treatment.

Micro-fragments from different treated surfaces were sampled and analyzed. Untreated surface samples were also collected as control. The collected samples, type of treatment and reaction times used for the complete removal of black crusts are listed in Table 1.

Table 1. Analyzed samples.

Sample	Type of Treatment	Number of applications and reaction times
TZ CRc	Untreated area (solid black crust sample)	-
TZ CHb	Solid black crust sample treated with **Surfactant**	3 appl. of 22 h
TZ BIOc	Solid black crust sample treated with **Micro4Art**	7 appl. of 22 h
TZ CH BIO	Solid black crust sample treated with **Surfactant+Micro4Art**	1 appl. of 2 h (Surfactant) + 2 appl. of 22 h (Micro4Art)

3.1.3 Evaluation of biocleaning assays

Biocleaning assays carried out on the funeral monument are globally effective in the removal of black crusts, while displaying some differences.

The Micro4Art treatment used alone or with Tween20 was more selective for the gypsum component with respect to the treatment with the surfactant alone. Moreover, at microscopic level, the result obtained with the treatment using only bio-formulation and with the surfactant, a certain heterogeneity was observed; this effect was not visible when the combined treatment was done. Another advantage of the combined effect of surfactant + bio-formulation is the reduction in application time and duration, conferring considerable economic benefits, as well as respecting the artwork. Indeed, a long-term application of aqueous packs on stone substrates may have negative effects on the artwork's conservation.

3.1.4 Restoration of the artwork

In accordance with the results obtained from the biocleaning tests and with the *Soprintendenza*, a proper restoration strategy was designed for the entire statue surface (Figure 1), and was performed by the restorer Dr. Eleonora Gioventù. The restoration procedure consisted of different steps, summarized as follows:

- removal of dust and micro-suction;
- stone surface cleaning and removal of black crusts using a Tween20 pack and following application of Micro4Art-sulfates bio-formulation, provided by Micro4yoU;
- removal of base stucco and reintegration;
- surface and aesthetical trimmings.

Sulfate removal from mural paintings of the Queen Theodolinda Chapel in Monza Cathedral

The object of the experimentation was the pictorial cycle decorating the Queen Theodolinda Chapel situated in Monza Cathedral (Figure 2). The artwork was subjected to an intervention of mild conservation, involving the most prestigious Italian research Institutes[1] and important sponsors. The experimentation carried out until now is not exhaustive and is still in progress and is intended to be a first innovative approach to the delicate issue of sulfate removal from mural paintings.

Figure 1. Upper body of the statue of Anna Zuccari, pen name Neera. On the left: before restoration; on the right: after restoration.

3.2.1 Artwork description and state of conservation

The pictorial cycle "*Delle Storie della Regina Teodolinda"* painted by "La Bottega degli Zavattari", was started in the first half of 1400 and completed in 1446, as stated in the contract. It is composed of 45 scenes which narrate episodes of life at court, with a precious description of clothes and armours, presenting an extraordinary description of courtly life in 15th century Milan.

The artwork was almost entirely realized using colors diluted in organic media, sometimes using milk of lime as a basis or, very seldom, the "*buon fresco*" technique. This technical choice has allowed for a particular chromatic vivaciousness through considerable recourse to gilding carried out with the method of "*pastiglia a rilievo*" ("tablet relief") using gypsum and glue, whereas the dresses, damasks and armours are made of golden or silver tin foil and veiled with copper resinate and red lacquer. At the same time, the choice of the organic binders is in itself the cause of extreme weakness, exacerbated by decay owing to ageing and manifold past restoration interventions.

Figure 2. Pictorial cycle decorating the Queen Theodolinda Chapel situated in Monza Cathedral.

The trial with sulfate-reducing bacteria was carried out on two scenes belonging to the pictorial cycle of the Queen Theodolinda Chapel: Scene 16 (upper rectangle in Figure 2) and 33 (lower rectangle in Figure 2).

Scene 16 belongs to the group describing the preliminaries to the wedding of Theodolinda and Autharis. It represents the episode in which Autharis is informed of Theodolinda's arrival in Lombardy by the delegates (Figure 3a). This scene, which has never undergone cleaning, was characterized by exfoliation and blistering of the pictorial film, caused by previous sulfation, still ongoing at the moment of treatment.

Scene 33 describes the apparition in Theodolinda's dream of the Holy Spirit, in the form of a dove, suggesting the place where she should build her church (Figure 3b).

In this scene the superficial salts appeared in a crystalline form, previously treated for sulfate removal by means of packs of sepiolite, deionized water and ion-exchange resins.

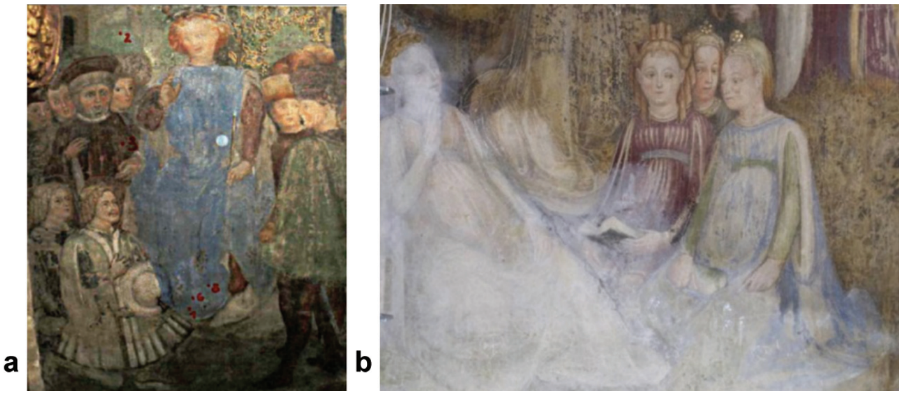

Figure 3. Scene 16 (a) and 33 (b).

3.2.2 Experimental setting and results

For sulfation removal, Micro4Art-sulfates were used in a freeze-dried form; before application, it was rehydrated with deionized water to the concentration of $5´10^7$ cells/ml. Two different thickeners were selected for the installation of the packs: Carboneutralgel and Arbocel BWW40 (Bresciani Srl).

With respect to Scene 16 (Figure 4), initially, a test application was performed in order to check the compatibility of the pack and time contact with the surface. According to the results and due to the frailty of the pictorial film, successive tests were carried out using two Japanese paper sheets with a short contact time (approximately 2 hours), in order to avoid chromatic alteration and to enhance the result of the application.

On the Lapis lazuli surface of Autharis' tunic, a preliminary cleaning step was carried out using deionized water, then the left hem of the tunic was subjected to Micro4Art-sulfate treatment using Carboneutralgel as thickener. Two samples (sample A and B) were collected before and after biocleaning, respectively.

In the following table (Table 2) the results of the chemical analyses carried out on sample A and B are reported.

Table 2. Results of chemical analyses carried out on sample A and B of scene 16.

Scene	Sample	Chlorides (mass %)	Nitrates (mass %)	Sulfates (mass %)
16	A) on the Lapis lazuli of the left hem of Autharis' tunic. Before biocleaning, after a soft water-cleaning.	0.08	0.41	3.20
	B) on the Lapis lazuli of the left hem of Autharis' tunic. After biocleaning by means of Micro4art-sulfates in Carboneutralgel (Bresciani).	0.01	0.12	0.75

In Scene 33 the superficial salts appeared in a crystalline form (Figure 3b). After a mechanical dusting, the complete area was repeatedly rinsed using deionized water across the Japanese paper. An extracting action through a pack of Sepiolite and Arbocel BWW40 (2:1), applied by spatula on two Japanese paper sheets and left on the painting until completely dry (about 15 hours), followed. Afterwards this area was repeatedly rinsed. Once dry, observation of the surface showed a thinning of the saline patina, but not its complete removal.

After pre-consolidation treatment of the Lapis lazuli painting of the background, by calcium-hydrate nanomolecules applied using a brush, two other neutral extracting packs as mentioned above were carried out. The area was then treated by means of anionic ion-exchange resins, applied over two Japanese paper sheets and left until completely dry (about one hour) (sample 4, Figure 4). Sulfation removal gave good results, as proved by the percent of sulfates, which was very high before treatment and 0.36% after treatment (sample 4, Table 2).

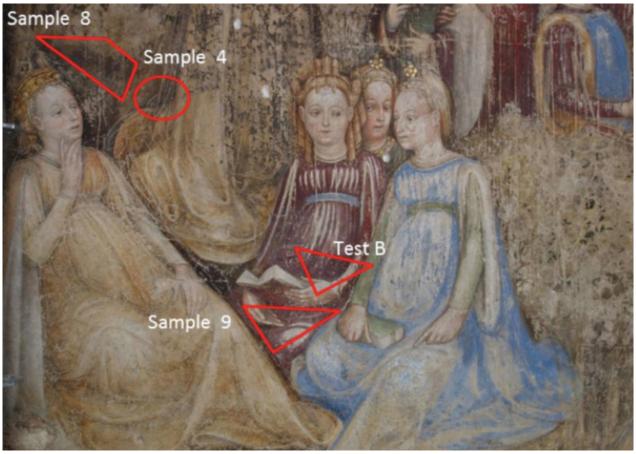

Figure 4. Scene 33 with the areas treated using different methodologies (in red).

The area of vermilion, in the mantle of one of the women, indicated as sample 9, was treated with Micro4Art-sulfates in Arbocel BWW40 (Figure 4). In this area too, the pictorial film remained intact and the chemical analyses recorded the lowest sulfate values: 0.19% (mass percentage).

In the following table (Table 3) the results of the chemical analyses carried out on sample 4 and 9 are reported.

Table 3. Results of chemical analyses carried out on sample 4 and 9 of scene 33.

Scene	Sample	Chlorides (mass %)	Nitrates (mass %)	Sulfates (mass %)
33	Before treatments*	ND	ND	>2
	4) on ground vegetation, above the tail of the dress of Theodolinda in the act of prayer. Treatment by means of packs of sepiolite, Arbocel, deionized water, ion-exchange resins.	0.20	2.06	0.36
	9) on the vermilion dress of the woman holding a book. After treatment with Micro4Art-sulfates supported on an Arbocel BWW40 tablet.	0.20	1.33	0.19

Due to their high solubility and deliquescence (property of some hygroscopic substances which pass in solution through absorption of water vapor from the environment, holding humidity), chlorides and nitrates constitute very hazardous salts for stone materials. For this reason, their acceptability limit is very low and near to the instrumental detectability limit. Instead, for sulfates, tolerability is higher.

In the following table (Table 4) the tolerability of these salts is reported:

Table 4. Acceptability limits for chlorides, nitrates and sulfates on stone materials constituting cultural heritage.

	Low	Significant	High
Chlorides	0.01 - 0.09 %	0.10 - 0.99 %	>1 %
Nitrates	0.01 - 0.09 %	0.10 - 0.99 %	>1 %
Sulfates	0.10 - 0.99 %	1 - 2 %	>2 %

Nevertheless, this work should be considered a “first step” in the employment of sulfate-reducing bacteria for the restoration of mural paintings, which constitute a very complex category of art works. More research must be conducted in order to verify the compatibility of the method with different kinds of materials, such as pigments containing metals (e.g. copper and lead), which could be susceptible to the hydrogen sulfide released by sulfate-reducing bacteria, or pigments with oily binders, based on malachite and copper resinate, which are very delicate. Moreover, another interesting issue to analyze is exposure time.

According to the results obtained from the Ionic Chromatography analyses, an interesting consideration can be made on the reduction of nitrates after bacterial application. The mass % of nitrates has passed from 0.41 to 0.12 in Scene 16 and from 2.06 to 1.33 in Scene 33. These data can be correlated to the nitrate-reducing ability of sulfate-reducing bacteria, already discussed in previous studies. In fact, *D. vulgaris* possesses the proteins FprAs which provide constitutive protection against nitric oxide exposure [10, 12]. Moreover, SRB of the genus *Desulfovibrio* are able to carry out dissimilatory nitrate reduction to ammonia thanks to the presence of *NrfA*, a gene encoding for a nitrite reductase. *NrfA* plays an important role in those strains able to use nitrate and nitrite as an electron acceptor when sulfate is not freely available and in those bacteria capable of reducing nitrite but not nitrate [9, 11]. Further investigations and trials for the removal of nitrates using sulfate-reducing bacteria could be interesting.

On account of this, the effectiveness of Micro4Art-sulfates in the removal of nitrates can be considered another interesting and promising result.

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Notes

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Summary

In the last decades, the contribution of different scientific disciplines in the field of restoration and conservation of cultural heritage to finding alternative methods of investigation that are even more effective and fully respect artworks, operators and environment, has greatly increased.

An example is the University of Milan patent that provides for the use of some specialized bacteria as cleaning agents. This method, known as *biocleaning*, is based on the use of *Desulfovibrio vulgaris*, a sulfate reducing bacterium, for the removal of sulfate alterations from stone surfaces.

Recently, the university spin-off Micro4yoU purchased the patent by initiating a series of investments aimed at enhancing the commercial product from prototype.

The present work describes: the desulfation mechanism operated by *Desulfovibrio vulgaris* and the technological shift necessary to obtain a biological formulation usable *in situ*, with two practical case studies.

Riassunto

Negli ultimi decenni è cresciuto il contributo che le diverse discipline scientifiche hanno dato al recupero e alla conservazione del patrimonio artistico per trovare soluzioni sempre più efficaci e rispettose del bene, dell'operatore e dell'ambiente.

Un esempio è un brevetto sviluppato dall'Università degli studi di Milano che prevede l'utilizzo di alcuni specifici batteri come agenti di pulitura. Tale metodo, indicato col termine di bio-pulitura propone, tra gli altri, l'impiego del batterio solfato riduttore *Desulfovibrio vulgaris* per la rimozione di alterazioni solfatiche da superfici litoidi.

Recentemente, il brevetto è stato acquisito dallo spin-off universitario Micro4yoU che ha avviato una serie di investimenti per sviluppare, a partire dal prototipo di laboratorio, un prodotto commerciale.

Nel presente lavoro verranno illustrati: il meccanismo di desolfatazione ad opera di *Desulfovibrio vulgaris*, il processo di trasferimento tecnologico affrontato per ottenere un formulato microbico utilizzabile in cantiere e due casi studio applicativi.