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Biotechnology applied to cultural heritage: biorestoration of frescoes using viable bacterial cells and enzymes

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ABSTRACT

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Aims: To set up and employ, for the biorestoration of cultural heritage (altered frescoes), an advanced and innovative biotechnology method based on the sequential use of whole viable bacterial cells and specific enzymes.

Methods and Results: The bioremediation intervention consisted of the direct application onto an artwork surface of whole bacterial cells of the *Pseudomonas stutzeri* A29 strain (bioaugmentation), followed by, in a final step, a purified *Protease* enzyme. The bioremediation was performed on a Spinello Aretino fresco that had become altered by the animal glue residues of past restoration. For the reader's interest the fresco is the 14th century *Conversione di S. Efisio e battaglia* (Conversion of S. Efisio and battle), size 3·5 × 7·8 m at the Pisa Camposanto Monumentale, Italy. An assessment was made of the final costs of the biological tests (whole bacterial cells, enzymes) so as to compare them with other intervention techniques.

Conclusions: A successful innovative biological approach to recover valuable frescoes was set up, and the best conditions for treatment efficiency were identified. Furthermore the cost of the biological cleaning using viable bacterial cells and enzymes (*P. stutzeri*, *Protease*, *Collagenase*, 1 : 3 : 10, ratio respectively) was much lower than that of other conventional methods, making this biotechnology not only very interesting but also very competitive.

Significance and Impact of the Study: New biotechnologies with an innovative, soft approach to the 'biocleaning' and 'biorestoration' of cultural heritage are in constant demand, and our results are clear evidence that such an approach has been achieved; the technique could be of significant importance towards developing other goals.

Keywords: bacteria, biocleaning, biorestoration, enzymes, frescoes, gas chromatography coupled with mass spectrometry (GC–MS) and pyrolysis/GC–MS analyses.

INTRODUCTION

Outdoor artwork, especially where lithoid materials, stones, frescoes and paint are involved, is very susceptible to deterioration that is brought about by changes mainly caused by ageing and, in recent decades, to pollution. In fact, in urban areas, damage because of the increased pollution of today is manifested on monument surfaces by alterations such as black crusts, nitration, sulphation and the deposition of dust and residual hydro-carbons (Saiz-Jimenez 1995).

In addition to air pollutants, the surfaces of man-made artistic stonework can also be altered by organic matter that has been applied, but then not completely removed, during restoration; in many cases this can pose serious danger even to the preservation of the artwork itself (Ranalli *et al.* 1996, 2000). In fact such 'leftover' compounds often act as a good growth substrate for micro-organisms and mycetes that

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destroy the surface, and allow hyphae penetration to some depth.

Moreover, the process of detaching frescoes from walls prior to restoration calls for notable quantities of organic compounds (such as glue and casein) that become distributed on both the painted surface and at the back of the fresco.

This was the condition of the Pisa frescoes (in the Monumental Cemetery, Camposanto) that cover a surface area of about 1500 m^2; these frescoes date back to the XIV century and are by famous painters such as Antonio Bonaiuti, Antonio Veneziano, Benozzo Gozzoli, Taddeo Gaddi, Francesco Traini, Bonamico Buffalmacco and Spinello Aretino.

Most of the frescoes had been restored using traditional chemical and physical techniques in which residual organic substances and salts are removed by ammonium carbonate solution and organic solvents. However even today's enzymes, surfactants and solubilizing agents have failed to clear away past restoration material and restore some of the frescoes (Makes 1988; Bellucci and Cremonesi 1994; Bonomi 1994; Wolbers 2000) that today exhibit a particular hydrophobic behaviour related to weathering and protein polymerization.

In order to choose suitable conservation practices, and to resolve the above-mentioned problems, it is most important to identify both the original organic materials applied and those belonging to restoration work, and also materials that have undergone transformation through natural ageing and pollution. As artwork samples are obviously unique and generally very small, and the compounds to be determined are in low concentration, it is fundamental to use sensitive and selective techniques like gas chromatography coupled with mass spectrometry (GC-MS) and pyrolysis/GC-MS (PY/GC-MS) (Colombini *et al.* 1999, 2003).

Up until this 'real' work on the frescos we had only used viable micro-organisms to degrade and remove substrate supported organic matter under laboratory conditions (Ranalli *et al.* 2003a). Thus we had to first identify the unwanted organic matter on the frescos, and verify the difficulty of removing such organic compounds by traditional methods. We then began experimenting the use of viable bacterial cells to degrade the organic matter, i.e. using them as 'biorestoration' agents on the frescoes; as the organic matter degraded the residues were 'rinsed off' and removed by enzymatic treatments.

The present research was aimed at dissolving the adhesive (animal glue) between the painted fresco face and the adhering gauze, and thus allow the gauze to be detached. We took the following steps:

i the characterization of the organic adhesive matter on the gauze used to detach the fresco from the wall: GC-MS and PY/GC-MS analytical procedures identified the natural organic compounds and their degradation products
ii the developing and improving of an innovative biorestoration system, that we had already set up on a laboratory scale, to remove the organic matter from the fresco surface.

We also assessed the costs of the whole bacterial cell and enzyme biotechnologies, and compared them with traditional methods.

MATERIALS AND METHODS

Micro-organisms, media and cultural methods

We tested *Pseudomonas cepacia* strain DSMZ 7288 (Deutsche Sammlung von mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), *P. testosteroni* strain DSMZ 1455, *P. fluorescens* strain DSMZ 304, *P. flavescens* strain DSMZ 12071, *P. stutzeri* strain A 29 (DISTAM-DISTAAM Strains Collections, University of Milan–University of Molise, Italy), *P. stutzeri* strains DSMZ 5190, and ATCC 23856 (American Type Culture Collection, Rockville, MD, USA). To select the best culture to apply to the altered fresco surfaces we assessed the biodegrading activity of the culture by adding animal glue at 0·5% and 1%, supplied as the complex organic matter and energy sources in the mineral medium M9, under aerobic conditions, at 10, 20, 28 and 37°C (Ranalli *et al.* 1996, 2003a). Microbial growth was monitored by $O.D._{560}$, and an assay was made of ATP content and the viable cell count in plate count medium. The selected strains were stored on standard plate count agar (Difco).

Preparation of the bacterial suspension for application

Suspensions containing exponentially growing bacteria, *ca* 10^8 CFU ml^{-1}, were obtained by inoculating 3 ml of an overnight broth culture into 300 ml fresh broth medium and incubating it in a shaker (200 rev min^{-1}) for 24 h at 28°C. The cells were centrifuged at 7000 *g* for 10 min at 4°C, washed twice with phosphate-buffered saline, and re-suspended in sterile 0·8% (w/v) NaCl solution, pH 7·2; the final cell concentration was above 10^8 cells ml^{-1}, corresponding to an $O.D._{560nm}$ of *ca* 1·6; the cells were used immediately or stored at 4°C during conservation and/or transport from the laboratory to the field application.

A fully computerized laboratory batch fermenter (20 l useable volume) (mod. Biostat C, B. Braun; Biotech International, Helsungen, Germany) was adopted to obtain, when needed, a relevant amount of viable bacterial cell biomass. The best selected bacterium for the *in situ*

biorestoration was cultivated, at the same concentration as the cultures, for 24 h on broth medium containing animal glue as the complex organic matter. The cells were recovered '*in vitro*' by centrifugation and re-suspended in NaCl solution at a final abundance of $1–3 \times 10^8$ cells per ml.

Whole bacterial cell applications on a fresco

Viable cells of the selected strain were applied to the fresco surface at *ca* 20°C and 10°C to evaluate the effect of temperature on the removal of organic matter. The bacterial culture was applied in three ways to test the application effect: (i) spraying, (ii) brushing and (iii) a covering cotton wool layer soaked with bacterial suspension.

In the first case a manually operated atomizer (useful volume 0·5 l) was used to spray the fresco surface from a distance of 25–30 cm, distributing the bacterial cells regularly by consecutive left-right and top-bottom dispersions; to favour distribution, large fresco areas were subdivided into areas to form rectangular reticules (treated areas *ca* 200–400 cm^2).

For the brushing distribution we adopted a flat paintbrush (8–12 cm wide) to deliver the cells manually, making gentle brush strokes to deliver the suspension over all the surface in a regular layer and taking great care to avoid detaching altered pellicles.

In the third application type we employed the same brushed-on cell distribution (as in application 2) and then covered the fresco with a thin layer (0·5 cm depth, 10 cm wide, 10–20 cm long) of hydrophilic sterile white cotton wool wet with the same bacterial cell suspension.

Monitoring of viable microbial cell counts

Throughout the biorestoration of the fresco, we constantly evaluated the total viable microbial cell numbers. For application 3 this was carried out on small samples taken from the cotton layer (*ca* 1·0 g), using the plate counts technique in standard plate count agar (Difco), incubation for 48 h at 28°C expressed as CFU g^{-1}. In applications 1 and 2, when the microbial count was determined directly on the fresco surface, we adopted the replica-plating technique; the results are expressed as CFU per unit area. Sterile velvet was pressed onto the 'cleaned surface' of the fresco, and then onto plate count agarized medium. Finally, in order to verify microbial viability during bacterial cell application, ATP assays (total and free) were carried out using the sterile swab technique (Ranalli *et al.* 2003b).

Enzyme treatments

Enzyme treatments were performed after the bacterial cell applications had removed as much glue as possible.

Five pure enzymes (*Collagenase* Type IA and Type V from *Clostridium histolyticum*, 1–3 U mg^{-1}, pH 7·5–8·0; *Protease* Type XIV from *Streptomyces griseus*, 4 U mg^{-1}, pH 7·5–8·5; *Protease* Type XIX from *Aspergillus sojae*, 0·4 U mg^{-1}, pH 8·4; *Lipase* Type VII from *Candida cylindracea*, 400–900 U mg^{-1}, pH 8·4–8·8; Sigma-Aldrich, St Louis, MO, USA) were tested, both separately and mixed, for the removal of the weathered glue.

Purified enzyme solutions were added delicately to the altered fresco surface by three different techniques: (i) swab, (ii) paper-disc and (iii) brush.

Enzymatic activity

The levels of enzymatic activity of the pure bacterial cultures were determined using the API-ZYM^{TM} system (bioMérieux Italia, Rome, Italy). The specific galleries (nm° 5, 11, 12) were inoculated with 65 μl of 10^{-1} adequate suspension ($O.D._{560\ nm}$ of ca 1.1) of 1 ml of an overnight broth culture (at 12th hour) to 9 ml of sterile solution (NaCl 9·0 g l^{-1}). After incubation for 4 h at 20, 28 and 37°C, the galleries were activated by adding 30 μl of Reagent ZYM A and Reagent ZYM B (bioMérieux) and after 5 min we assigned values ranging from 0 to 5 in relation to the colour developed in each enzymatic reaction, using the colour chart provided by the manufacturer (Principi *et al.* 2003). For the purposes of this study, the results are reported as reactions of low intensity (1), moderate intensity (2–3) and high intensity (4–5) (Tiquia 2002).

The grade of enzymatic activity on native animal glue and on animal glue cross-linked was determined using the Feller test (Feller *et al.* 1985; Cremonesi 1999).

ATP determination

Total ATP assays to monitor both culture growth on the laboratory scale, and the viability of the bacterial cell suspensions during the *in situ* biorestoration processes, were performed using a specific enzymatic kit (NRM/Lumit-QM, code 9332-l; Lumac B.V., Landgraaf, the Netherlands). A Biocounter 1500 P luminometer (Lumac B.V.) equipped with a photomultiplier tube set at 7200 RLU with 200 pg ATP in 100 μl of Lumit buffer and Lumit-QM reagent was used (Ranalli *et al.* 1998, 2003b).

Microscope observations

Microbial growth and cell survival were determined by optical microscope (Axiophot, Zeiss; LEO Elektronen-mikroskopie GmbH, Oberkochen, Germany) and scanning electron microscopy (SEM) observations throughout the application of the biocleaning treatment to the altered

surface of the fresco. The samples for SEM observation were treated overnight in a solution of 2% glutaraldehyde (0·01 mol l^{-1} phosphate buffer), and then immersed in 1% osmium tetroxide. A microscope operating at 10 kV was used (Zeiss DSM 940A; LEO Elektronenmikroskopie GmbH).

Chemical analyses

Reagents. All the solvents were Baker HPLC grade. Hexadecane and tridecanoic acid were used as internal standards (IS). Hexamethyldisilazane (HMDS) and *N*,*O*,bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane were purchased from Sigma (Milan, Italy), *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) with 1% trimethylchlorosilane was from Fluka (Milwaukee, WI, USA); all were used without any further purification. Standard solutions of amino acids in HCl 0·1N, norleucine (IS2) and hexadecane (IS1) as injection internal standard were purchased from Sigma-Aldrich.

Analytical procedure based on selective extraction and hydrolysis assisted by microwave and GC-MS analysis. The glue sample (50–100 μg) was extracted with ammonia solution, subjected to microwave-assisted acidic hydrolysis (microwave oven model MLS-1200 MEGA Milestone; FKV, Sorisole, Italy), and the resultant hydrolysed solution derivatized with MTBSTFA and GC-MS analysis (6890N GC System Gas Chromatograph coupled with a 5973 Mass Selective Detector; Agilent Technologies, Palo Alto, CA, USA) to determine the protein content. The residual of the ammonia extraction was subjected to saponification, extraction with *n*-hexane, acidification and extraction with diethyl ether to obtain, respectively, the neutral and acidic fractions, subsequently analysed by GC-MS after derivatization with BSTFA to determine the presence of natural resins, lipids and waxes. The detailed procedure is reported in the literature (Colombini *et al.* 1999, 2003; Bonaduce and Colombini 2003).

Analytical procedure based on PY/GC-MS. A few μg of samples together with 5 μl of a HMDS solution (20% in acetone) were pyrolysed at 600°C in a pyrolyser operating at constant temperature mode Pyrojector II (SGE, Austin, TX, USA). Experimental conditions are reported in detail in previous work (Colombini *et al.* 2003).

Mass spectra assignment. Mass spectra assignment was generally based on direct matching with spectra from the Wiley 275 library: spectra were retained when the correlation match index was >95%. We compared pure compounds whenever possible.

Physical analyses. The thickness of the glue layers was measured at different points of the fresco using a laser sensor micrometer, Thrubeam LX2-V model (Keyence Corp., Osaka, Japan).

Spinello Aretino fresco

During World War II the frescoes belonging to the complex of the Monumental Cemetery of Pisa were ruined by a bomb (1944) but, after detachment from the walls, were restored at a later date. From among them we chose, for the present study, the XIV century fresco, *Conversione di S. Efisio e battaglia*, painted by Spinello Aretino; the surface area is *ca* 50 m^2 (26 m^2 treated) and the contained inorganic pigments are known from relative documentation.

Unfortunately the restoration techniques and conservation conditions were inappropriate, and 'our' fresco had soon shown alteration phenomena such as swelling and detachment of the painting pellicle. In the 1980s, the fresco was again detached from the walls at Camposanto using 'tear off' techniques that involve, on site, the application of a wide gauze directly onto the fresco surface, using animal glue as the consolidating agent; thus the gauze and fresco become one layer that is then detached from the wall. The back of the fresco is reinforced by a canvas cloth, applied using casein, which is, in turn, supported on asbestos, again with casein.

One of the greatest difficulties during the restoration was the detachment of gauze still adhering, from the previous restoration, to the paint layer on the front surface; this was despite soaking with solvents. Over the years the glue had altered greatly, and had become very hard and resistant to commonly used solvents. Our task was to dissolve the animal glue on the paintwork itself to release the gauze residues without removing the casein supporting the back of the fresco.

The conservation state was also worsened by the contraction of the glue and the swelling of the casein at the back; furthermore different synthetic organic substances had been used in the past restoration and these had favoured the polymerization of the compounds, making their removal an onerous task.

RESULTS

Biological treatment

Selection of the strain for application in the bioresto-ration. Table 1 shows the results of the laboratory scale trials of bacterial culture growth rate at 28°C on broth medium containing animal glue as the complex organic matter.

There were marked growth rate differences among the tested *Pseudomonas* species and the *P. stutzeri* strains; *P. stutzeri* A 29 showed the highest cell density (1·8 $O.D._{540}$ and >8·5 log CFU ml^{-1}), a relevant ATP content, higher

Table 1 Growth yields in minimal medium with added glue, assessed by $O.D._{560}$ and CFU at 36th hour, at 28°C; ATP content, protease activity at 10, 20, 28 and 37°C and microscope observations at 12th hour

Bacterial culture	$O.D._{560}$ (±S.D.) (36 h)	Log CFU ml^{-1} (36 h)	Total ATP (RLU)* (12 h)	Protease activity (trypsin)† (°C)				Microscope observation‡ (12 h)
				10	20	28	37	
P. cepacia DSMZ 7288	1·5 (0·15)	<8	++	1	3	3	4	++
P. testosteroni DSMZ 1455	0·1 (0·1)	<3	+/−	0	0	0	0	+/−
P. fluorescens DSMZ 304	1·45 (0·2)	<8	++	0	2	3	3	++
P. flavescens DSMZ 12071	1·4 (0·15)	<8	++	0	2	3	4	++
P. stutzeri A 29 (DISTAM-DISTAAM, Italy)	1·8 (0·2)	>8·5	++++	2	4	5	5	+++
P. stutzeri DSMZ 5190	1·65 (0·15)	8	+++	1	3	4	4	+++
P. stutzeri ATCC 23856	1·7 (0·2)	8	+++	1	3	4	5	+++

RLU, relative luminose unit.

*+/−, very low; ++, low; +++, high; ++++, very high.

†Overnight broth-culture at 12th hour: reaction of low intensity (value of 1: 5 nm of hydrolysed substrate); moderate intensity (value of 2–4: 10–30 nm of hydrolysed substrate); high intensity (value of 5: ≥40 nm of hydrolysed substrate).

‡Presence of bacterial cells per field (+/−, very rare; ++, low; +++, high; ++++, very high).

enzymatic protease activity and, from optical microscope observations, the greatest number of bacterial cells per field.

The influence of temperature on the growth of the selected bacterial strains was investigated by cultural, enzymatic and microscopic tests, the response of *P. stutzeri* strain A29 growing cells being noted at 10, 20, 28 and 37°C. Cell density increased slightly when the incubation temperature was maintained under 10°C for 36 h, and improved a little at 20°C (data not shown). However at constant temperatures of 28°C and 37°C there was a very marked increase in cell growth at 24 and 36 h, with abundant biomass production. Moreover, notable increases in enzymatic activity were observed at the tested temperatures: in fact, the response of the protease reaction in *P. stutzeri* strain A29 on overnight broth culture at 12th hour, showed low intensity (value of 1) at 10°C, moderate intensity (value of 2–4) at 20°C, and high intensity (value of 5) at both 28 and 37°C (Table 1).

Figure 1 shows the SEM observations on the selected *P. stutzeri* strain A29 cells.

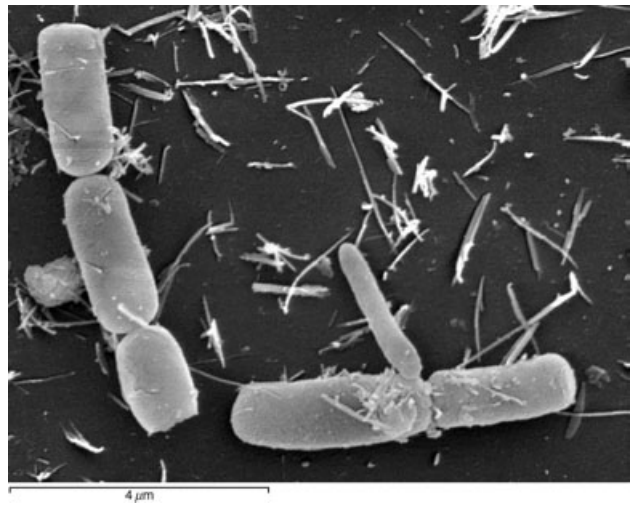

Fig. 1 Scanning electron microscopy of *Pseudomonas stutzeri* strain A29 cells in 0·8% NaCl solution

Chemical analyses

The GC-MS and PY/GC-MS results from several fresco samples (1–5 mg) evidenced both animal glue, used to glue the gauze onto the front of the fresco, and casein which came from the back of the painting. The PY/GC-MS identified, by specific markers, the proteins indole for casein and pyrrole and alkylpyrroles for the animal glue. The GC-MS amino acidic profiles showed that all the proteins had undergone degradation. This was particularly evident in the samples taken from the front of the fresco (Fig. 2) where there was a typical collagen protein pattern; the samples from the back of the fresco showed a high degree of degradation in the casein amino acidic pattern, because of natural ageing and micro-organism attack (Fig. 3 in GC-MS). In fact, this phosphoprotein had a low content of amino acids like methionine, phenylalanine and lysine. These results concord with those of samples from other analysed frescoes (Colombini *et al.* 1995, Colombini *et al.* 1999). Moreover, as protein recovery was *ca* 20% w/w of the sample weight, it would appear that cross-linking reactions had occurred.

This hypothesis was also confirmed by the presence of formalin, which had been added to the glue mixture to avoid

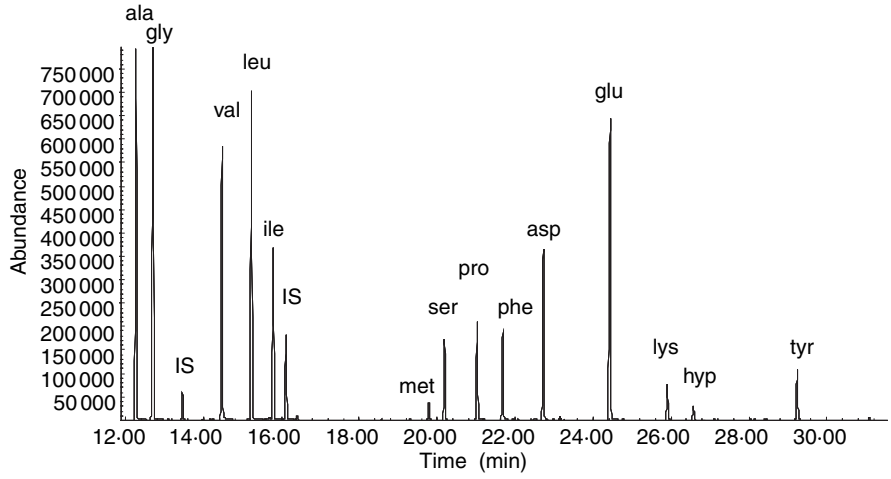

Fig. 2 GC–MS chromatogram (acquired in the SIM mode) of a sample taken from the front of the fresco. Internal standards (IS), alanine (ala), glycine (gly), valine (val), leucine (leu), isoleucine (ile), methionine (met), serine (ser), proline (pro), aspartic acid (asp), glutamic acid (glu), lysine (lys), hydroxyproline (hyp) and tyrosine (tyr)

fermentation in alkaline conditions; furthermore, formalin in the presence of casein during drying gives rise to insoluble compounds that are difficult to remove.

The relevant amounts of sulphates, phosphates and oxalates were because of the eternit, that together with the lime putty contributed to the sulphation of the frescoes, to both the phospholipids of the casein and the final products of the oxidative degradation of the animal glues and casein, and to metabolic micro-organism degradation.

These salts were responsible for the whitish patina observed on the surface of almost all the frescoes.

Our results have revealed that the painted surface of Spinello Aretino's fresco is embedded in a hard net of polymerized and cross-linked proteins which, in turn, are glued to the canvas.

Physical analyses

The thickness of the glue layer was demonstrated to vary from 1 to 3 mm corresponding to *ca* 4 mg cm^{-2}.

Biorestoration treatment with whole bacterial cells

The *ex situ* biorestoration treatments were performed by distributing a suspension of viable precultured bacterial cells of *P. stutzeri* strain A29 on the altered fresco.

Of the three application modes, the best results were achieved by putting a cotton wool layer soaked with microbial suspension onto the fresco surface. In fact, in terms of humidity preservation, this technique resulted in constant, and favourable, microenvironmental conditions between the viable cells and the fresco surface throughout the biological treatment (time of contact and adhesion). The spraying and brushing cell applications produced unfavourable rapid and excessive drying, with a consequent reduction in bacterial cell viability and activity. Furthermore, in both cases it was also necessary to apply further cell suspension aliquots from time to time after the first application, placing an extra burden on our resources with regard to microbial biomass availability, and on the restorers from the point of view of labour. Thus, on the basis of these findings, the bacterial culture mode of

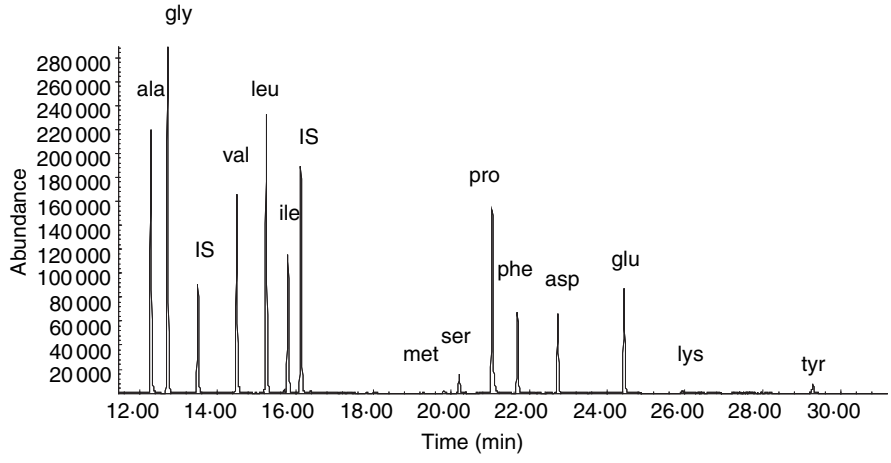

Fig. 3 GC–MS chromatogram (acquired in the SIM mode) of a sample taken from the back of the fresco. Internal standards (IS), alanine (ala), glycine (gly), valine (val), leucine (leu), isoleucine (ile), methionine (met), serine (ser), proline (pro), aspartic acid (asp), glutamic acid (glu), lysine (lys), hydroxyproline (hyp) and tyrosine (tyr)

application adopted was the culture-soaked cotton wool layers (40 cm wide, 6·0 m long); these were placed over the whole fresco surface, guaranteeing homogeneous conditions and saving time during application and recovery.

Different tests showed the marked influence of temperature on the biological treatment. An environmental temperature below 10°C resulted in no evident biological effects, however at *ca* 20°C the collagen began to dissolve 8–24 h after the daily cell suspension application; this did not happen in the control areas.

At 28°C the cellular activity was so intense, just 8–12 h after applying the bacterial suspension (log 8 CFU g^{-1} that means *ca* 100 million of viable cells per g), that the treated areas were 'clean and free' from the animal glue without any structural damage.

Nevertheless the degree of glue removal depended on the thickness of the glue layer at the start of the treatment, varying between 80 and 100%; some areas were cleaned completely, while others (where the glue was as thick as 3 mm) were left with residues, but never greater than *ca* 0·5 mm.

When residues were still present after the treatment it was found that prolonging the bacterial treatment gave the possibility of obtaining complete removal, but there was some risk to the fresco. In fact, for periods of longer than 15–17 h, the prolonged contact with the moist cotton led to the swelling and detachment of paint fragments.

Figure 4 shows the removal of cotton layer enriched with *P. stutzeri* strain A29 after 10-h biorestoration from *ex situ* fresco altered by animal glue (*Conversione di S. Efisio e battaglia*, XIV century) at Pisa Camposanto Monumentale (Italy).

Fig. 4 Removal of cotton layer enriched with *Pseudomonas stutzeri* strain A29 after 10-h biorestoration from *ex situ* fresco altered by animal glue (*Conversione di S. Efisio e battaglia*, XIV century) at Pisa Camposanto Monumentale (Italy)

Table 2 Enzymatic activities on native animal glue and on animal glue cross-linked (altered fresco)

	Native animal glue		Animal glue cross-linked	
Enzymes	Specific activity	Feller test	Specific activity	Feller test
Collagenase Type IA	5*	+++	2*	+
Collagenase Type V	4*	+++	1*	+/−
Protease Type XIV	4†	++	1†	+/−
Protease Type XIX	5†	+++	2†	+
Lipase Type VII	1‡	+/−	0‡	+/−

Assay of specific activity: *chymotrypsin; †trypsin; ‡lipase
+/−, very low; +, low; ++, middle; +++, high.
Reaction of low intensity (value of 0–1: 0–5 nm of hydrolysed substrate); moderate intensity (value of 2–4: 10–30 nm of hydrolysed substrate); high intensity (value of 5: ≥40 nm of hydrolysed substrate).

Biorestoration treatment with enzymes

After removing the gauze through bacterial activity, small organic matter residues (the glues themselves) still remained on the surface. These were treated by enzyme solutions.

The purified enzymes, *Collagenase* Type IA and *Protease* Type XIX, used separately and in mixture (data not shown here), showed the highest removal efficiency, both on native animal glue and on animal glue cross-linked (fragment of altered fresco), as reported in Table 2.

However, because of the high cost of the collagenase-based enzyme, we adopted *Protease* Type XIX (1·0 g/100 ml^{-1} Tris buffer solution, pH 7·8–8·2, at 38°C, for 10–15 min).

During application a portable heating device was used to maintain optimal temperatures for the fresh enzyme solutions (38°C). Throughout the fresco biorestoration process and during the enzymatic treatment, the recorded indoor environmental temperature showed an average of 28 ± 3°C.

Of the three different enzyme application modes, swab, paper-disc and brush, enzyme distribution was best by brush; in fact its efficiency in removing residual organic matter and animal glue was probably favoured by the gentle pressure of the bristles of the brush and the repetitive and gentle mechanical pressure of the manual application.

Monitoring of the biorestoration process

An important aspect of bioremediation is the process monitoring. This was performed initially on the laboratory scale using a microbiological approach. Instead the *in situ* application called for more rapid, although not always specific, techniques such as the determination of ATP content by bioluminescence.

During the biorestoration process on a full scale fresco we assessed how time and the environment affected the

dynamics of *P. stutzeri* A29 bacterial cell survival, measured as log CFU ml^{-1} and ATP content and compared with a control. The results from a repeated series of tests showed that there were no significant statistical differences in the first 12 h and 14 h of the biological process. However a significant variation appeared in the parameters of the 16 and 24-h treatments using the *P. stutzeri* strain A29. The recovered cells (fresco surface) showed a final mean value in cell viability of log 4–log 5 CFU cm^{-2}, compared with log 8 CFU cm^{-2} in the control set (initial fresco surface enriched by viable bacterial cell suspensions). The total ATP content showed little variation as a function of treatment duration. In fact, at hours 6, 10 and 12, the ATP content was comparable with the initial values, whereas at 16 h, it was slightly lower than the control.

The pH values were continually monitored by a probe located between the fresco surface, in direct contact with the altered patina, and the cotton wool layer enriched with the selected bacterial cell suspension. After 12 h of biorestoration, only a slight reduction in pH value was noted (from 7·2 to 6·7).

At the end of the microbial cell cleaning of the fresco surface, and after removing the remaining bacteria by a final washing with distilled sterile water (three soft manual sponge applications), microbiological analyses were made on the fresco surface to check for the possible presence of residual microbial cells. The lack of growth on the inoculated plates of the replica-plating technique confirmed that no microbial cells were detectable with the methods used. The data were confirmed by the free and total ATP content values.

Figures 5 and 6 show the effects of 'biocleaning' and 'biorestoration' process obtained with *P. stutzeri* strain A29 cells and *Protease* enzyme on *Conversione di S. Efisio e battaglia*, fresco (XIV century) at Pisa Camposanto Monumentale, (Italy) (top and bottom, before and after treatment respectively).

Fig. 5 Effects of 'biocleaning' and 'biorestoration' process obtained with *Pseudomonas stutzeri* strain A29 cells and *Protease* enzyme on *Conversione di S. Efisio e battaglia*, fresco (XIV century) at Pisa Camposanto Monumentale, (Italy) (top and bottom, before and after treatment, respectively)

Economic evaluation of the biorestoration

An analysis of the costs of the bioremediation process shows that the use of bacterial cultures is more convenient economically than the use of enzymes like *Protease* and *Collagenase*.

In fact, the total cost can be quantified as being *ca* 50 l^{-1} for bacterial culture, 150 l^{-1} for the *Protease* and 500 l^{-1} for the *Collagenase*, using a litre of the bacterial or enzymatic suspension for *ca* 2 m^{2} of fresco surface. Thus the cost ratio was 1 : 3 : 10.

On comparing the costs of the bacterial biorestoration process and the more frequently used chemico-physical techniques, it is evident that the latter are less convenient; this can be seen in terms of both the total time needed for the restoration and the number and duration of the application times (repetitive treatments every 6–8 h) as well as in the greater costs involved for specialized personnel, restorers and finally environmental safety.

DISCUSSION

The success of the viable bacterial cell application, compared with that of enzymes is, presumably, attributable to the wide versatility of the bacteria and their activity. Bacteria are known to produce not only constitutive but also inducible enzymes that attack and degrade different types of molecules. The synthesis of inducible enzymes takes place only in the presence of a substrate, creating a regulatory effect. Thus the use of micro-organisms is more effective than just the use of a single enzyme that attacks only specific linkages. In fact, the alkaline properties of formalin, a common component of the organic matter, are conducive to polymerization, protein cross-linking and reticulation during drying, resulting in the formation of insoluble compounds.

Fig. 6 Effects of 'biocleaning', 'biorestoration', process obtained with *Pseudomonas stutzeri* strain A29 cells and *Protease* enzyme on *Conversione di S. Efisio e battaglia*, fresco (XIV century) at Pisa Camposanto Monumentale, (Italy) (top and bottom, before and after treatment, respectively)

Thus in the case of frescoes where the glue and casein compounds are protein weathered, and altered by the presence of formalin, the effectiveness of the micro-organisms is really important as the enzymes, which are highly specific, may not be useful. Moreover, the constitutive and induced enzymes of viable cells of versatile bacteria like *Pseudomonas* are able to break up many organic compound chemical linkages.

These heterotrophic bacteria were applied to the Spinello Aretino fresco as it was known that Spinello used only inorganic pigments; thus his frescoes would not be damaged in any way during the short application time of the bacterial solution.

Many factors such as temperature, relative humidity, pH, carbon and energy sources, etc. can influence metabolic activity. Thus, to optimize the metabolic efficiency we expected on the fresco, we had to check these factors accurately in the laboratory, our assumption being that if biorestoration could not be achieved under optimal conditions on the laboratory scale, it would be unlikely to succeed in an uncontrolled environment.

Thus we had to verify whether our successful laboratory results would be confirmed under real, on site, environmental conditions, especially for outdoor artwork.

In artwork bio-remediation, bacterial treatment requires the application, by spray, brush or compress, of selected micro-organisms to the artwork surface. The way the application is carried out depends on the type of alteration, the artwork material, the location of the areas undergoing treatment, and the metabolic activity of the selected microflora (aerobic and anaerobic).

Indeed the effectiveness of the biological process is defined by the time course of the treatment. Long treatment times require a high biomass concentration and favourable contact between the micro-organisms and the surface. Consideration must also be given to an adequate cell-carrier support; in the case of 'our' fresco the choice was cotton wool. A previous laboratory study carried out on stone artwork had shown the best support matrix for the bacteria to be sepiolite (Ranalli *et al.* 2000).

All artwork biorestoration requires a final, accurate cleaning phase. Indeed, at the end of specific biological processes all residual microflora should be removed carefully to avoid the continuation of undesired metabolic processes. Thus an adequate strategy for artwork protection must be planned; if the micro-organisms remain alive and active they can cause material loss or damage, either directly or as a consequence of their catabolic by-products.

The proposed biological technique, that constitutes an accurate cleaning phase, was a fundamental 'must' for the side of the fresco called 'intelaggio' (the painted side), although long contact with a water solution could have posed an indirect risk to the safety of the fresco. In this phase it was very important to reduce to the minimum both the volume of water addition and its contact time on the fresco surface.

Possible damage because of prolonged bacterial treatment has, till now, only been evidenced by Ranalli *et al.* (2000), who discussed the removal of black crusts from stone. In this case, sulphate-reducing bacteria reduced the sulphates and, consequently, removed the crusts; however prolonged contact between the bacteria and the stone caused the precipitation of sulphide salts. Thus it is very important to control the duration of the application.

Nevertheless, when artwork undergoing microbial remediation has peculiarities that permit other forms of intervention, adequate surfactant solutions and weak biocides can be used.

In order to identify optimal conditions to develop a bioremediation approach for the use of selected microflora on artwork, it is most important to monitor the microbial activity during, and at the end of, the biological process.

Monitoring techniques are routinely used to carry out microbial counts based on culturing methods and direct counts by microscope observation. In fact SEM and optical microscope observations give important information on the presence of microflora and their degree of adhesion to the surface of the treated materials. Rapid analyses are fundamental to the monitoring of biorestoration processes, and fast feedback in the monitoring of microbial activity can be achieved through bioindicators like ATP content by low light imaging and dehydrogenase activity (DHA) (Ranalli *et al.* 2000, 2003a).

The greatest advantage of bacterial/enzymatic bioresoration, compared with traditional methods (chemical, physical and mechanical), is that this new method is not destructive and removes only extraneous substances or altered compounds from the fresco. Moreover, microbial cultures (bacteria) have non-specific activity, while the use of enzymes is highly specific and limited. Another advantage is the use of safe micro-organisms (not pathogenic bacteria or yeasts, not spore-forming bacteria) for both the operators and the environment. Finally, the evaluation of costs and convenience has revealed the transferability of this technological innovation to other fields outside cultural heritage.

Organic matter (i.e. collagen) on the surface of altered frescoes can be removed, under appropriate conditions, by treating the fresco surface with selected aerobic heterotrophic microbial cultures (bioaugmentation) and/or selected pure enzymes. As a consequence of the bacterial activity, carbon dioxide is released from the pollutants without the use of toxic compounds, so the method must be considered environment-friendly.

After optimizing the parameters for the biological process on the laboratory scale, and subsequently checking the results on real outdoor samples, we consider that the application of the method in wider fields would require: (i) an abundant activated biomass produced under environmentally controlled conditions; (ii) a rapid and sensitive method to monitor the biological activity and avoid undesirable effects; (iii) the correct and complete removal of residual biological activity.

We are presently researching ways of applying, *in situ*, biological methods to several works of art, and to reduce the limitations and possible risks of such methods. Our results have confirmed the potential of biorestoration processes as soft innovative biotechnology. Such restoration, aimed at the recuperation of degraded artwork, is nondestructive, and uses micro-organisms and their metabolic activity in association with purified enzymes.

The positive results we have achieved have led the Technical Commission for Restoration (Pisa, Italy) to accept and approve the use of a *P. stutzeri* cell culture suspension and purified *Protease* enzyme solutions for biological applications; thus the innovative 'biorestoration' bioaugmentation process is already being used.

The biorestored fresco discussed here is an important case study on the real, true to life scale where soft biotechnologies can play a positive role.

We foresee that appropriate micro-organisms will be used for the remediation and recovery of artistic stonework altered by atmospheric pollutants like nitrogen and sulphur oxides, compounds that rapidly accelerate artwork deterioration.

It has been shown that cultural heritage restoration now has a new and innovative technology: biorestoration through the use of viable cells of selected bacteria; such restoration poses no risk at all to human health, or the environment, and has shown great potential for removing undesirable compounds from the surfaces of works of art.

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