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ORIGINAL ARTICLE

Bioremoval of sulfates from black crust: a case study of St. Augustine Tower, Goa-India

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Abstract

Cultural heritage (CH) comprises physical artifacts, paintings, stonework, traditional activities, frescoes, monuments, and other buildings that are vital to be preserved for future generations. Exposure of these to the effects of environmental factors, including sulfation, microbial cell lysis, primary colonization, etc., leads to their biodeterioration. Sulfation is a significant problem due to the formation of black crust on heritage structures and artwork surfaces. Conservation of stonework itself is a challenging task. To date, chemicals, solubilizing agents, and surfactants are used to remove contaminants and residual matter likely to menace the monument. Bioremediation technologies have now evolved as a green approach to removing sulfates, which employ suitable microbial cultures capable of utilizing sulfates when provided with the appropriate carrier. The objective of this research involves screening and isolating Sulfate-reducing bacteria (SRB) and testing the effectiveness of isolated SRB in removing black crusts from the selected area of the chosen heritage site, namely St. Augustine church. We emphasize the significance of isolated SRB and the use of an appropriate carrier for sulfate metabolism on transformed stone surfaces. The ability of the delivery system to guarantee water availability for microorganisms, no toxic impact on the environment, no negative impact on the surface treated, easy preparation, application, and removal from stone surfaces after the treatment are all critical. We report on the ability of potential strains of *Pseudomonas azotoformans* strain PgBE29 and *Arcobacter ellisii* strain LMG 26155 in consortia to bio-clean sulfate from the black crust, including architectural documentation, characterization of the alteration, the efficiency of the method proposed, and an assessment in terms of cost-effective technology and sustainability towards the environment.

Keywords Biorestoration · Black crust · Cultural heritage · Consortia · Sulfate-reducing bacteria

Introduction

Millions of tourists visit historical sites, museums, galleries, and archaeological digs every year, and the preservation of our heritage is essential to understanding our past. The conservation and restoration of cultural heritage (CH) is thus of paramount significance to heritage caretakers. It is crucial to preserve cultural heritage, as climate change and pollution make it more vulnerable to deterioration. Any treatment considered should adhere to the International Charter of Conservation. Currently, our cultural heritage is disappearing faster than in any other instance of civilization. It is vital to comprehend the fundamental mechanisms contributing to the deterioration of historical sites and artifacts.

One of the studies involving the remediation of biodeterioration in Demetra and Cronos focused on deteriorated sculptures, and through microscopic observations and

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chemical analyses, it unveiled two reported alterations: biological discoloration and black crust (black, green, and green-black). The chemical studies revealed that the apparent chemical modifications were black crusts consisting primarily of gypsum. In addition to the transition of calcite to calcium sulfate, calcium nitrate may arise due to calcite interacting with NOx (nitrogen oxides) in the atmosphere (Ricci et al. 2006).

Sulfate components combine with oxygen in the environment to generate sulfur dioxide, which oxidizes to sulfur trioxide. Sulfur trioxide is transformed into sulfuric acid when it encounters moisture. The rise in rainwater acidity over the past years has caused eventual harm to nature, sculptures, and monuments where it reacts with calcium carbonate/lime ($CaCO_3$) coated onto the surface of monuments, forming calcium sulfate/gypsum ($CaSO_4$) (Driesschе et al. 2017). Deterioration occurs in the form of black crusts due to gypsum crystallization when pollutants from the air, like particles containing carbon, are enclosed in the matrix, causing blackening in these areas and forming black sulfates containing crust (Del Monte et al. 2001). In other words, the common phenomenon of blackening on the surfaces of monuments is due to high air pollution, which transforms insoluble $CaCO_3$ into calcium sulfate dehydrate ($CaSO_4{\cdot}2H_2O$) (Chapoulie et al. 2008; Stocks-Fischer et al. 1999; Tiano 2002; Tiano et al. 1999; Warren et al. 2001). The reaction occurs as follows (Yadav et al. 2020; Constantin et al. 1996).

$$2SO_2 + O_2 \rightarrow 2SO_3$$

$$SO_3 + H_2O \rightarrow H_2SO_4$$

$$CaCO_3 + H_2SO_4 \rightarrow CaSO_4.H_2O + CO_2$$

The exquisite stoneworks are seen outdoors in an open setting, which accelerates the deterioration process owing to the atmospheric effect (Fernandes 2006; Li et al. 2008). Environmental elements that contribute to this degradation include humidity, temperature changes, inorganic wastes, wind, pH, and rainfall. For researchers around the world, restoring historical buildings and architectural monuments is an aspect of greatest significance (Dakal and Arora 2012). None of the possible remediation methods, including physical and chemical treatments, proved adequate for removing the blackened crust from the monument frescoes (Cappitelli et al. 2006; Ranalli et al. 2019). Generally, microbes have deleterious impact on stone; however, recent studies demonstrate that microorganisms can be employed to remove detrimental compounds from the artwork (Cappitelli et al. 2006, 2007; Gorbushina et al. 1993, 2002; Liberatore et al. 2012; Pinna 2022; Yesankar et al. 2022; Elsayed et al. 2023; Jiang et al. 2023). Bioremediation is the technique of introducing microorganisms or nutrients to a contaminated location in order to speed up the natural biodegradation processes. The benefits of bioremediation include its low cost, environmental friendliness, and green nature, which make it more popular with the general population as a whole.

Bio-cleaning technologies have been used in the last few decades to eliminate undesired substances, including organic and inorganic residues from stone surfaces of cultural heritage, ceramics, a material made up of paper, and concrete materials using specific bio formulations with *Pseudomonas sp.* and *Desulfovibrio sp.* (Gauri et al. 1989; Cappitelli et al. 2006, 2007; Alfano et al. 2011; Troiano et al. 2013; Barbabietola et al. 2012). The first investigation involved immersing marble fragments in cultures of *Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans* for 60–84 h, demonstrating removal efficiencies of 40–100% for sulfates from the black crust (Gauri et al. 1989).

Following these investigations, it was found that submerging huge, delicate artworks in liquid culture has numerous disadvantages; hence, a delivery mechanism was investigated in the following studies to minimize damage caused by this method's water absorption to stone. Therefore, bio-treatments for the removal of sulfate have been carried out, consistently in anaerobic conditions using a delivery system composed of inorganic sepiolite carriers augmented by *D. vulgaris* and *D. desulfuricans.* Sepiolite was employed; however, occasionally this resulted in dark residues in some cases. Furthermore, sepiolite as a carrier did not ensure adequate water availability for the chemical changes in cells during application on the stone's surface. As a result, for the trapping of *D. vulgaris ATCC* 29,579 cells, the inorganic carrier was replaced by hydrobiogel-97, an organic carrier (Cappitelli et al. 2006). The feasibility of achieving an appropriate sulfate removal in a shorter amount of time was shown by laboratory-scale testing using artificially enriched specimens and original altered stone pieces (Ranalli et al. 1997).

More recent research demonstrated enhanced carriers such as agar-base gel with *Pseudomonas stutzeri DSMZ 5190* strain to eliminate salt precipitates on the Church of Santos Juanes frescoes in Valencia, Spain. The in situ bioapplication was performed using carbogel as a carrier at Vicenza, Italy, applied as a thin layer to establish the availability of very little free oxygen, and recited three times after 15 h to remove sulfates (Cappitelli et al. 2006, 2007; Martino et al. 2022; Wu et al. 2022). Another illustration of such a study involves a marble-base artifact from Milan, Italy, where *D. vulgaris ATCC 29,579* successfully bio-cleaned the sulfated crust utilizing carbogel as a carrier. Carbogel has been used successfully in several bio-cleaning treatments as a carrier to remove the black crust with sulfates (Cappitelli et

al. 2006; Alfano et al. 2011; Cappitelli et al. 2007). For the purpose of removing secondary iron black deposits, bacterial cells were cultured in a modified broth without iron, and anaerobiosis procedures ranged from 24 to 30 h.

Black crusts from the aged stone surface on Failaka Island, Kuwait, were removed by hydrobiogel-97 colonized with *D. vulgaris* (Elhagrassy and Hakeem 2018). *D. desulfuricans*, an anaerobic sulfate reducing bacteria (SRB), was the first microorganism to be tested as an approach to reducing sulfates and impurities in black crusts on marble. *D. vulgaris* and *D. desulfuricans* have successfully restored old masonry and artifacts since that pioneering effort (Ayangbenro et al. 2018). In the sulfate reduction process by SRB, Ca^{2+} ions are released from the calcium sulfate and react with the atmospheric carbon dioxide, which is converted to $CaCO_3$ (Silva-Castro et al. 2015). The process of crystallization occurs by encompassing ionic reactions, diffusion, and dissolution processes (Anbu et al. 2016).

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

According to Cappitelli et al. (2006), a number of strains, including *D. desulfuricans ATCC 13,541, D. vulgaris ATCC 29,579*, and *D. desulfuricans ATCC 29,577*, exhibit the potential to reduce sulfate. An anaerobic environment is a requirement for these organisms to grow. Since studies indicate that SRB may be able to remove sulfates from wastewater even before adopting the anaerobic biogas production process, SRB was isolated from the digestate of the biogas plant for this experiment (Van den Brand et al. 2018). As a result, the digestate from the biogas plant was used to isolate SRB serving as facultative anaerobes and identified as *Pseudomonas azotoformans* strain PgBE29 and *Arcobacter ellisii* strain LMG 26155. They have been applied as a result of their ability to successfully remove sulfates from the dark crust that develops on the surface of monuments. The approach lies in using a consortium of SRB entrapped in a carrier. Various delivery agents, including sepiolite, arbogel, agar, cotton wool, carbogel, and hydrobiogel-97, can be used. But when all the delivery agents were compared, carbogel emerged as the most feasible selection due to its low cost and simplicity of use. It promotes strong interaction between the underlying substrate and the cells, thus prolonging the longevity of bacterial cells. Additionally, it encourages their removal following treatment (Andreolli et al. 2020).

This study focuses on the Church, of Our Lady of Grace, universally known as St. Augustine's church in Old Goa, on the hilltop, Goa, India. It is surrounded by the Kadamba plateau to the south and the Mandovi River to the north at a distance, and it is owned by the Archaeological Survey of India (ASI) Goa Circle. This church belonged to the Augustinians, who arrived in Goa in 1572 and erected their monastery. Because there was no maintenance, the huge vault fell in 1842. Its facade and towers were destroyed in 1931 and 1938, respectively.

In 1986, the United Nations Educational, Scientific, and Cultural Organisation (UNESCO) recognized the St. Augustine Church as a world-historical site. As a result, the ASI prioritized the preservation of this cultural heritage property. Because most heritage sites are relatively large, the efficacy of the approaches utilized for architectural documenting should be addressed (Jo and Hong 2019; Fallahi 2008). This church's facades were primarily encased in black crust. In order to address a gap in heritage site preservation studies in Goa, our research focuses on the architectural documentation of the entire monument for simple comprehension of the exact location of experiments as well as restoration studies employing newly isolated strains of SRB.

Materials and methods

Architectural documentation

The selected site, namely, St. Augustine's Tower in Goa, is located on the west coast of India (15.500522253113065, 73.90646555665418 (15°30'01.8"N 73°54'22.7"E 15.500488, 73.906306). A preliminary visit to the site involved selecting an area for the experiment based on a visual inspection. The preparation of measured drawings was carried out in two methods. The overall area of the main altar was measured by a surveyor using a total station. This method gave us the location of the peripheral walls and levels from a common reference point. The measurements of the stone facade under direct experimentation were taken manually with the help of a measuring tape, and the technical drawing was prepared in AutoCAD. This drawing was the basis for all other pictures recording the areas of experimentation through the various stages of the experiment.

Sample collection from the facade of St. Augustine Tower for Scanning Electron Microscopy and Energy Dispersive X-ray spectroscopy (SEM-EDS)

Samples were collected by placing the polypropylene-based, pressure-sensitive tape on the black patina of the selected facade portion and removing it. To set control, samples were also collected from the unaffected facade region. The unaffected area is visually clean and displays no discoloration or black patina. These samples were then kept at 4 °C for further study. The collected samples on tape were cut into 1 cm × 1 cm, placed on the carbon grid, and sputter coated. The images were captured at 2,000 X to 50,000 X

magnifications using an EVO 18 ZEISS Scanning Electron Microscope, FEI, Quanta FEG 250, USA, at the SEM facility, Central Sophisticated Instrumentation Facility (CSIF), Birla Institute of Technology and Science Pilani, K. K. Birla Goa Campus.

Enrichment and isolation of consortium of SRB from digestate of biogas plant

A sample of biogas slurry from an anaerobic digester at a biogas demonstration plant at Headland Sada in South Goa, Mormugao, India, 403,804, was collected in a sterile glass bottle to enumerate SRB. Five milliliters of the slurry was then inoculated into a sulfate-reducing medium prepared in serum bottles tightly capped with rubber stoppers and aluminium crimp caps (Hi Media, Mumbai) containing 1 g/l of yeast extract, 0.2 g/l of magnesium sulfate ($MgSO_4$), 0.010 g/l of Di-potassium hydrogen phosphate (K_2HPO_4), 0.1 g/l of ammonium ferrous sulfate/Mohr's salt ($(NH_4)_2Fe(SO_4)_2.6H_2O$) and 10 g/l of sodium chloride (NaCl) under nitrogen-rich environment devoid of oxygen. Post enrichment, the consortium was transferred to Baar's medium (3.5 g/l of 60% Sodium lactate, 0.5 g/l of ammonium chloride (NH_4Cl), 1 g/l K_2HPO_4, 2 g/l of $MgSO_4.7H_2O$, 1 g/l of $CaSO_4$, trace amounts of Mohr's salt in tap or distilled water) and Starkey's medium (3.5 g/l of 60% sodium lactate, 1 g/l of NH_4Cl, 0.5 g/l of K_2HPO_4, 2 g/l of $MgSO_4.7H_2O$, 0.5 g/l of Na_2SO_4, 0.1 g/l of $CaCl_2.2H_2O$ and trace amounts of Mohr's salt in tap or distilled water) (Toerien et al. 1968). The serum bottles were then incubated at 37 °C while purged with nitrogen gas to maintain anaerobic conditions. Post enrichment in specific media, the dominant sulfate-reducing bacterial strains were purified by streaking the cultures on sulfate-reducing medium agar plates and set in an anaerobic jar. The jar was purged with nitrogen gas to maintain anaerobic conditions and incubated at 37 °C.

16s rRNA sequencing of SRB

The cultures were purified by streaking the mixed culture on a sulfate-reducing agar plate and incubating it in an anaerobic jar at room temperature (RT) for 48 h. They were delivered to Eurofins Genomics India Pvt. Ltd. in Bengaluru, Karnataka, India, for 16 S rRNA sequencing (Butlin et al. 1949). The 16 S rRNA sequences from both cultures were used for identification using the National Centre for Biotechnology Information-Basic Local Alignment Search Tool (NCBI-BLAST), followed by phylogenetic tree construction using the fast minimum evolution method. The accession numbers for cultures were obtained by uploading the sequences to the NCBI nucleotide sequence database.

SEM of isolated bacterial cultures-*Pseudomonasazotoformans* strain PgBE29 and *Arcobacter ellisii* strain LMG 26155

The cells were grown in serum bottles in sulfate-reducing broth before being pelleted by centrifugation at 10,000 rpm for 10 min at room temperature. The cells were exposed to 1% glutaraldehyde overnight, followed by 0.1% aqueous osmium tetroxide for one hour (Ranalli et al. 1997). After washing with distilled water, the cells were treated for 10 min with increasing concentrations of ethanol ranging from 10%, 30%, 50%, 70%, 90%, and 99.9%, and then stored in 99.9% ethanol in a desiccator until SEM analysis. To boost the electrical conductivity of the sample surface, dehydrated samples were critical-point dried with carbon dioxide and sputter coated with a thin layer of gold. The images were captured at magnifications ranging from 2,000 X to 50,000 X with an EVO 18 ZEISS Scanning Electron Microscope.

Lab-scale biotransformation studies

Calcium sulfate/Gypsum crystals ($CaSO_4.2H_2O$) were artificially produced on Makranta marble by exposing the marble to 5 M sulfuric acid (H_2SO_4) for 1 h, followed by seven days of exposure to an open atmosphere (Giustetto et al. 2020). After seven days, the residue on the marble was analyzed for the formation of sulfates by SEM-EDS, and the sulfate reduction efficiency of consortia of *P. azotoformans* strain PgBE29 and *A. ellisii* strain LMG 26155 was investigated. Cells were grown in sulfate-reducing broth and centrifuged at 10,000 rpm for 10 min at RT. The supernatant was discarded, and the pellet was harvested and dissolved in phosphate-buffered saline (PBS) with a concentration of 8×10^4 cells/mL. The cells in PBS were then combined with an 8% carbogel carrier, forming jelly that was applied to the marble surface with a spatula and covered with moist tissue paper soaked in PBS. After 15-hour, the jelly-containing cells were removed, and the new cell sample was applied. This was done three times after fifteen hours. After drying the marble, the residue was analyzed using SEM-EDS to determine the elemental changes. To establish control, a portion of the marble was also treated with carbogel devoid of cells.

Preparation and application of the sample for bioremediation studies

This experiment was carried out on two panels with a black patina, each measuring 5 cm × 10 cm and marked in red (Fig. 1a). Similarly, areas for subsequent trials were delineated with string and measured with a simple hand-held

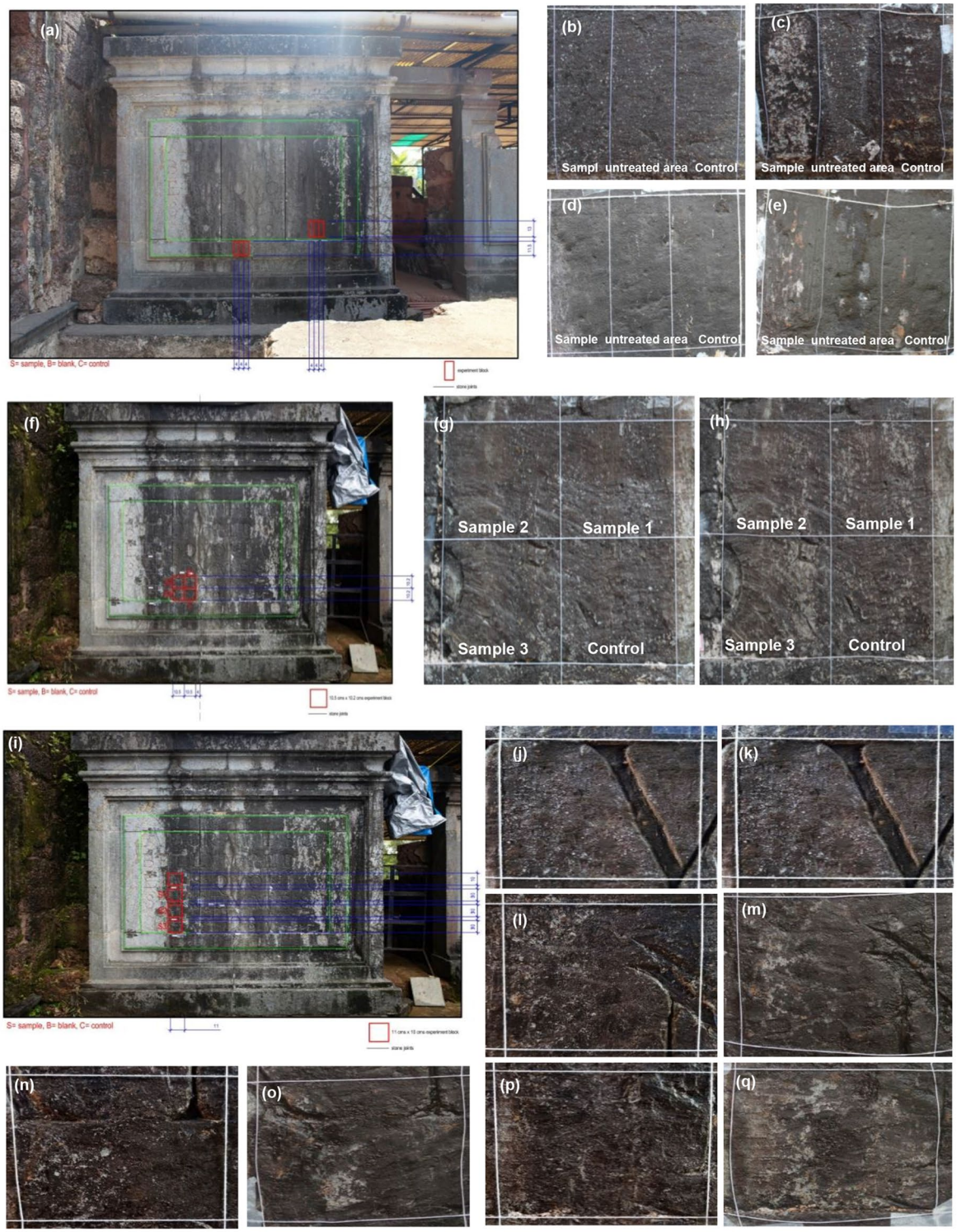

Fig. 1 (**a**) Marking of Site 1 and Site 2 under experimentation of test (**b**) Site 1 before application (**c**) Site 1 after application (**d**) Site 2 before application (**e**) Site 2 after application (**f**) Marking of site 3 under experimentation (**g**) Area before application (**h**) Area after application (**i**) Marking of site 4 under experimentation (**j**) Control before application (**k**) Control after five applications (**l**) Sample 1 before application (**m**) Sample 1 after five applications (**n**) Sample 2 before application (**o**) Sample 2 after five applications (**p**) Sample 3 before application (**q**) Sample 3 after five applications

measuring tape. AutoCAD was used to create a map of the discoloration on the facade, with (Fig. 2) as the base drawing overlay on a photograph. Before the experiment, the wall under investigation was protected from rain by a plastic shield that kept the wall dry. For the experiment, a wall section was divided into three parts. One section was treated with sulfate-reducing bacterial cells combined with carbogel, another with carbogel but no sulfate-reducing bacteria, and the third with carbogel alone. $(NH_4)_2Fe\ (SO_4)_2.6H_2O$ from the growth medium was eliminated to prevent iron precipitation under anaerobic conditions. During the exponential phase, the culture was harvested by centrifuging at 10,000 rpm for 10 min at 4 °C (Ranalli et al. 2005). The pellet was dissolved in PBS containing 0.408 g/l of potassium dihydrogen phosphate (KH_2PO_4), 0.522 g/l of K_2HPO_4, and 0.599 g/l of sodium lactate (Cappitelli et al. 2006) and stored at 4 °C until further usage. Bacterial cells in PBS were incorporated with the 8% carbogel carrier at 8×10^4 cells/mL (Bosch-Roig et al. 2015). It absorbs the suspension with regular stirring and produces a gel that offers slightly anaerobic conditions. Using a spatula, a 0.5-1 cm thick coating of carbogel mixed with bacterial cell suspension in PBS was applied to the targeted area of the St. Augustine wall and covered with PBS-soaked tissue. The temperature ranged from 28 to 35 °C during the experiment. The control was established by utilizing only carbogel without bacterial cells, and the untreated control area remained unaltered during the experiment (Cappitelli et al. 2006). After treatment, the carbogel-containing delivery system was removed with sterile swabs and hydrophilic cotton, and a new suspension of cells embedded in carbogel was administered after 15 h (Cappitelli et al. 2007). The procedure was repeated three times with a 15-hour gap between each application. Following the final removal of the sample, the clearance was visually examined, and images were taken using a Canon 1200D camera. In subsequent studies, the number of applications increased from three to five. The swab was taken in sterile saline, plated onto sulfate-reducing agar, and incubated at 37 °C to check for surviving cells on the monument surface.

Study of different cell concentrations optimum for sulfate removal by flow cytometry

P. azotoformans strain PgBE29 and *A. ellisii* strain LMG 26155 were cultured in a sulfate-reducing medium without iron. The culture was serially diluted up to 10^4 times with filtered MilliQ water. The optical density of each dilution was determined at 600 nm, followed by flow cytometry at the Department of Biological Sciences, BITS Pilani K. K. Birla Goa Campus, using Becton, Dickinson, and Company

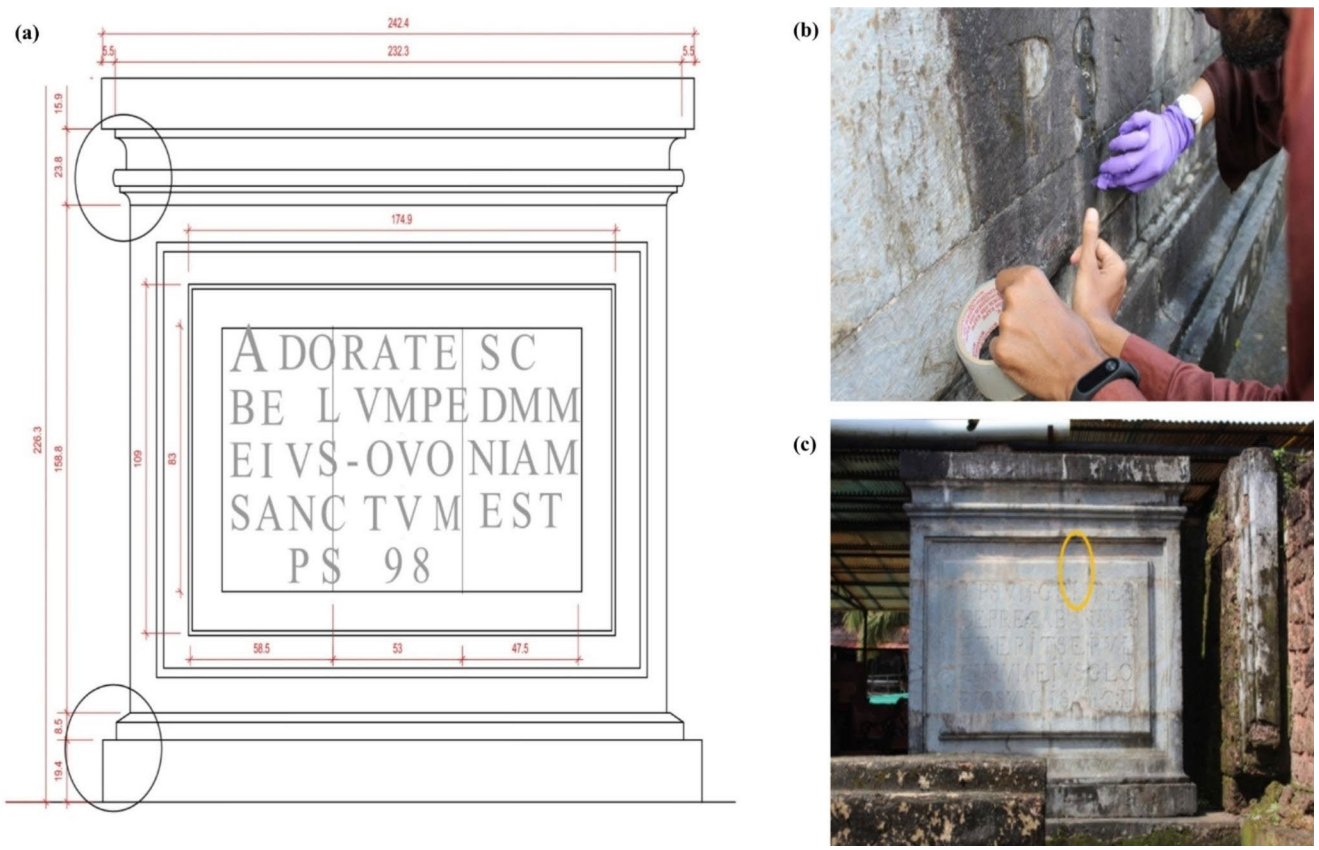

Fig. 2 (**a**) Measured drawing of Bassein stone panel under experimentation (**b**) collection of samples (**c**) wall with no black patina (Control)

FACS Melody, and Sr. No. R6617620170, USA. The flow rate was calibrated for flow cytometry analysis using filtered MilliQ water as a blank. The following formula yields the rate (R) in microliters per minute.

$$R = (Wi - Wf)\,(T^{*}d)\,,$$

Where Wi = initial weight (mg), Wf = final weight (mg), T = time (minutes), and d = density of the liquid used for calibration (distilled water = 1.00, seawater = 1.03).

Before performing flow cytometry analysis, dilution tubes were thoroughly vortexed, and 1 ml of each diluted sample was placed into the flow cytometry tube after measuring optical Density (OD) at 600 nm. The analysis duration was recorded, which is essential to determining cell concentration. Filtered MilliQ water was used as a reference, followed by dilutions with the start and end times of the measuring event documented. The absolute cell concentration for each population is calculated as follows: Where Cpop = concentration of population (cells/μL^{-1}), Npop = the number of cells acquired, T = acquisition time (minutes), R = sample flow rate (mL/min^{-1}), Vtotal = volume of sample plus additions (fixatives, beads, etc.) (mL), and Vsample = volume of sample (mL) (Marie et al. 2005).

$$Cpop = T^{*}NpopR^{*}\,(VtotalVsample)\,,$$

$$Correctto\,(withsubscriptsasset)\,:$$

$$Cpop = Npop^{*}(Vtotal/Vsample)/\,(R^{*}T)$$

The graph was plotted against OD v/s cell count, and the concentration of the corresponding diluted cultures employed in the experiment was determined. As shown in Fig. 1f, the wall space was divided into four 10.5 × 10.5 cm sections. Sample 1 was treated with the final cell concentration of 8×10^4 cells/mL; sample 2 was treated with 13×10^4 cells/mL, and sample 3 was treated with 21×10^4 cells/mL. The last one was left undisturbed and designated as an untreated control area.

Improving the efficiency of bioremediation by boosting the number of applications

The efficacy of *P. azotoformans* strain PgBE29 and *A. ellisii* strain LMG 26155 was tested in the following experiment by increasing the number of applications from three to five. The wall space was divided into four sections, each 10 cm × 11 cm, as shown in Fig. 6i. The area treated with carbogel devoid of cells was labelled carbogel and designated as control. Sample 1 was treated with a cell concentration of 8×10^4 cells/mL, Sample 2 with 13×10^4 cells/mL, and Sample 3 with 21×10^4 cells/mL. The cells were combined with an 8% carbogel carrier in PBS at the appropriate concentration. The jelly-containing cells embedded in carbogel were applied to the specified location using a spatula. The procedure was continued for 75-hours, with a 15-hour gap between each application. Following the final removal of the sample, the visible elimination of the black crust was noted.

Results

Architectural documentation

Architectural documentation determined the broad area of the main altar (as seen in Fig. 3). The main altar is oriented in the north east-south west (NE-SW) directions. It is approximately 16 m × 10 m. The area of experimentation marked 1 on the drawing is in the west corner of the main altar. Fig. 2 was used as a base drawing for further measurements of the exact location of experimentation (Beck 2013, Sabatini et al. 2000). The entire built structure with the bluish-grey Bassein stone where the area under experiment lies is 242.4 cm × 226.3 cm (height) × 87.5 cm (depth). The part of this face primarily affected by the black patina and directly under experimentation was the area with the inscription, which measures 147.4 cm (width) × 83 cm (height). This demarcation would ensure that the experiment would not be tampered with. A facade of engraved Bassein Stone on the west wall became our primary focus, displaying discoloration of varying intensities (La Russa et al. 2013). Further, there was a similar engraved Bassein Stone panel on the other extreme of this facade, which displayed no prominent discoloration. This would allow for comparison later if the experiment proved successful.

Sample collection from the wall of St. Augustine for SEM-EDS

The black crust sample collection from the patina can be seen in Fig. 2b. The sample recovered from the unaffected patina was clear white, as depicted in Fig. 2c. SEM-EDS analysis (Table 1) revealed the presence of sulfur (S) in the sample taken from the black crust along with microcrystalline gypsum with patchy crystals of calcite, a variable amount of iron (Fe), lead (Pb), carbon (C), aluminium (Al), manganese (Mn), silicon (Si), zinc (Zn), chlorine (Cl), potassium (K), particulate matter, and many other air particles from processes like combustion; however, the unaffected region, which was thought to be free of sulfates, did not reveal the presence of sulfates. SEM Images of the black crust aided in understanding conversion mechanisms and

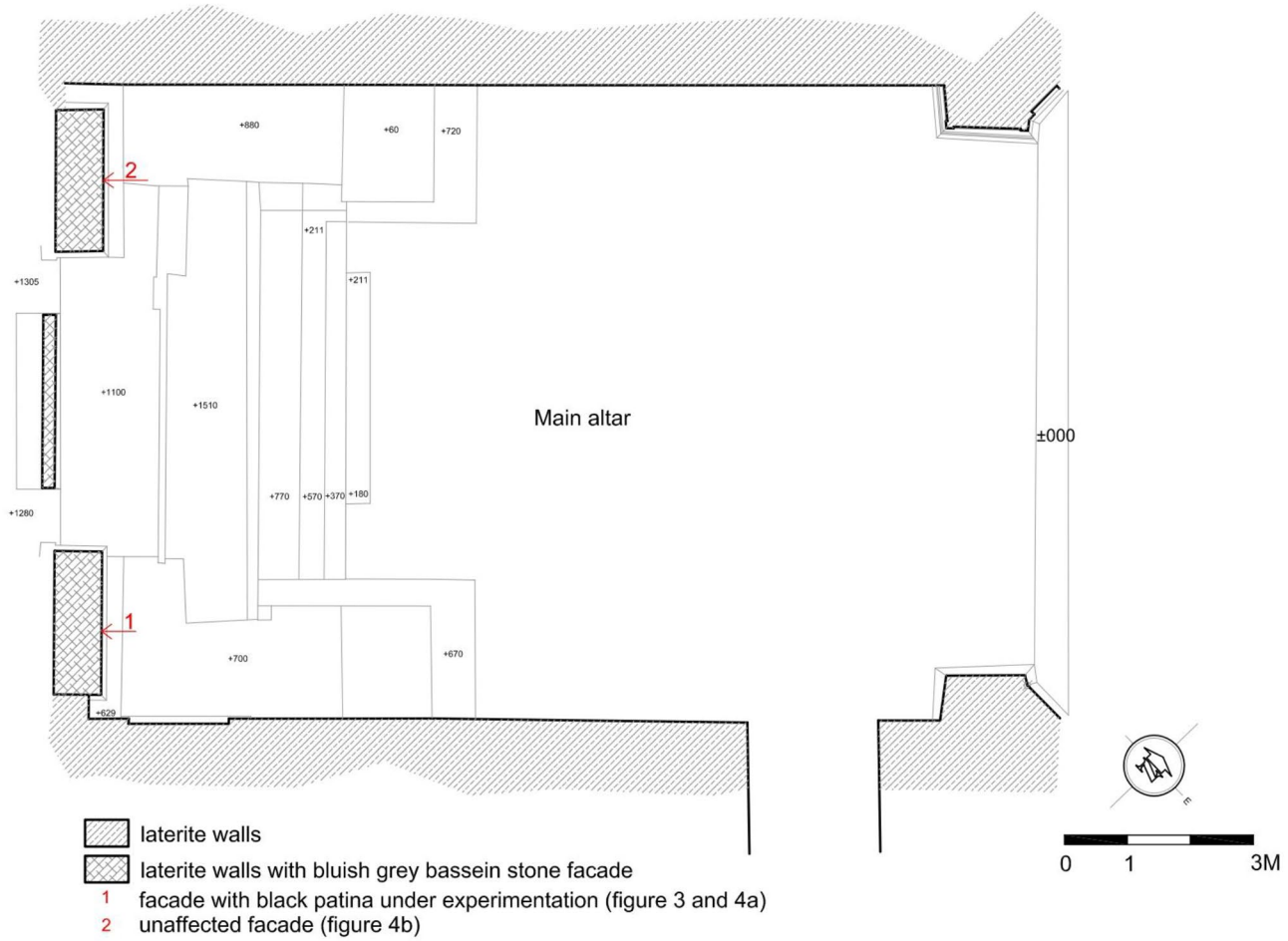

Fig. 3 Measured drawing of main altar showing location of facade under experimentation

estimating the degree of decay. They appear unsymmetrical, with short fissures and crystals of various sizes, forming a crust of varied thickness on the wall with non-uniform morphology ranging from 10 to 100 µm (Fig. 4).

Enrichment and isolation of SRB

The sulfate-reducing activity of the microorganisms was confirmed after a few days of inoculating slurry from the biogas plant into the medium. This inoculation resulted in the medium turning black due to the use of sulfates as terminal electron acceptors, reducing it to hydrogen sulfide (Suppl. Figure 1a). Following enrichment, culture transfers to Starkey's and Baar's medium showed that the media was black, indicating and confirming the predominance of sulfate-reducing bacteria in the enrichment culture (Suppl. Figure 1b and 1c). After purification, two distinctive colonies appeared on sulfate-reducing agar plates, indicating the presence of two discrete isolates in the medium.

Scanning electron microscopy and 16 S rRNA sequencing of SRB

SEM analysis of a consortium of SRB revealed the presence of cells that can be distinguished based on their shape. The cell morphologies discovered include bacilli and slightly curved rods ranging in size from 493 nm to 4 µm. (Fig. 5). Through 16 S rRNA sequencing, followed by NCBI-BLAST search, the purified cultures were identified as *Pseudomonas* and *Arcobacter* species. Phylogenetic analyses using the fast minimum evolution method revealed their evolutionary relatedness with other homologs shown in the figures (Suppl. Figure 2 and Suppl. Figure 3). They closely resembled *P. azotoformans* strain PgBE29 (99.42%) and *A. ellisii* strain LMG 26155 (99.13%). The sequences acquired for both cultures were submitted to the NCBI nucleotide sequence database under the accession numbers OP520922 and OP536157 for *P. azotoformans* strain PgBE29 and *A. ellisii* strain LMG 26155 respectively.

Table 1 Concentrations of elements (mean ± SD, n = 3) present in sample 1 and 2 (black patina) and sample 3 (control)

	Weight%			Atomic %			Net int.		
Elements	**S1**	**S2**	**S3**	**S1**	**S2**	**S3**	**S1**	**S2**	**S3**
C	28.600 ± 11.364	25.900 ± 11.578	0.960 ± 1.674	37.133 ± 11.767	34.466 ± 12.764	1.667 ± 2.887	18.333 ± 8.025	18.500 ± 13.201	0.733 ± 1.270
O	51.400 ± 1.682	51.030 ± 2.511	51.460 ± 3.963	51.167 ± 5.314	52.633 ± 3.855	65.533 ± 4.300	63.467 ± 26.980	57.533 ± 3.150	98.867 ± 77.192
Mg	6.630 ± 5.397	3.900 ± 3.672	16.430 ± 3.958	4.500 ± 3.651	2.833 ± 3.017	13.733 ± 3.060	21.467 ± 18.071	10.133 ± 8.997	53.733 ± 37.278
Al	1.800 ± 1.136	2.700 ± 0.458	0.930 ± 1.210	1.033 ± 0.666	1.667 ± 0.321	0.700 ± 0.889	5.533 ± 3.702	8.700 ± 1.453	4.033 ± 5.816
Si	9.400 ± 5.164	8.860 ± 5.733	17.560 ± 13.951	5.467 ± 3.201	5.500 ± 4.139	12.733 ± 10.191	38.667 ± 25.105	32.500 ± 17.708	66.133 ± 58.500
Mn	0.230 ± 0.404	0.000 ± 0.000	0.300 ± 0.520	0.067 ± 0.115	0.000 ± 0.000	0.100 ± 0.173	0.400 ± 0.693	0.000 ± 0.000	0.467 ± 0.808
S	0.030 ± 0.058	1.100 ± 0.794	0.000 ± 0.000	0.033 ± 0.058	0.567 ± 0.379	0.000 ± 0.000	0.133 ± 0.231	3.633 ± 2.380	0.000 ± 0.000
K	0.160 ± 0.289	0.160 ± 0.289	0.000 ± 0.000	0.067 ± 0.115	0.067 ± 0.115	0.000 ± 0.000	0.300 ± 0.520	0.533 ± 0.924	0.000 ± 0.000
Ti	0.130 ± 0.231	0.000 ± 0.000	0.000 ± 0.000	0.033 ± 0.058	0.000 ± 0.000	0.000 ± 0.000	0.200 ± 0.346	0.000 ± 0.000	0.000 ± 0.000
Fe	1.360 ± 1.350	1.900 ± 0.608	4.530 ± 1.290	0.400 ± 0.400	0.600 ± 0.265	1.667 ± 0.513	1.900 ± 2.193	2.633 ± 0.681	6.167 ± 1.234
Ca K	0.000 ± 0.000	2.230 ± 1.332	7.360 ± 12.759	0.000 ± 0.000	0.933 ± 0.681	3.800 ± 6.582	0.000 ± 0.000	5.233 ± 2.723	18.667 ± 32.332
Zn	0.000 ± 0.000	1.400 ± 2.425	0.000 ± 0.000	0.000 ± 0.000	0.400 ± 0.693	0.000 ± 0.000	0.000 ± 0.000	1.167 ± 2.021	0.000 ± 0.000
Cl	0.000 ± 0.000	0.260 ± 0.462	0.000 ± 0.000	0.000 ± 0.000	0.133 ± 0.231	0.000 ± 0.000	0.000 ± 0.000	0.733 ± 1.270	0.000 ± 0.000
P	0.000 ± 0.000	0.300 ± 0.520	0.000 ± 0.000	0.000 ± 0.000	0.167 ± 0.289	0.000 ± 0.000	0.000 ± 0.000	0.767 ± 1.328	0.000 ± 0.000
Cu	0.000 ± 0.000	0.230 ± 0.404	0.000 ± 0.000	0.000 ± 0.000	0.067 ± 0.115	0.000 ± 0.000	0.000 ± 0.000	0.167 ± 0.289	0.000 ± 0.000
PbM	0.000 ± 0.000	0.000 ± 0.000	0.400 ± 0.693	0.000 ± 0.000	0.000 ± 0.000	0.033 ± 0.058	0.000 ± 0.000	0.000 ± 0.000	0.667 ± 1.155

Lab-scale bio-transformation studies

SEM image (Fig. 6) shows the formation of gypsum as needle-like crystals on marble. The existence of C, O, Al, Si, S, Ca, Fe, Mg, and K was confirmed by EDS analysis. The results show that 4.9% of sulfur was present before treatment with a consortium of *P. azotoformans* strain PgBE29 and *A. ellisii* strain LMG 26155. Following treatment, the sulfur concentration was reduced from 4.9 to 0.56%, concluding that the *P. azotoformans* strain PgBE29 and *A. ellisii* strain LMG 26155 consortium might reduce sulfur, which was the rationale for picking these two prevalent sulfate reducers for sulfate biotransformation (Table 2).

Onsite experiment using SRB for Bioremediation

The jelly was effectively produced for the bioremediation experiment by adding a carbogel carrier to a *P. azotoformans* strain PgBE29 and *A. ellisii* strain LMG 26155 bacterial suspension in consortia. Following the application of the cells to the black crust, it can be seen (Fg.1c and Fig. 1e) that the part of the black crust treated with bacterial cells was cleared due to the utilization of sulfates by bacteria; however, there was no clearance in the control where only carbogel was used because there was no sulfate-reducing activity and the untreated control area was left untouched, so no change was observed. The findings on the wall enabled us to assess the efficacy of SRB in eliminating the sulfated black crust. This demonstrates that specific bacterial isolates are capable of sulfate reduction. The inconsistent clearing obtained is due to the unequal distribution of sulfates on the wall in their impure form.

Study of different cell concentrations optimum for sulfate removal by flow cytometry

The flow rate was determined using flow cytometry to be 16.33 ml/min. Cpop was determined for each dilution using flow cytometry analysis that recorded the number of events, followed by a graph of OD v/s cell count where three different concentrations were confirmed (Fig. 7). The use of varying cell concentrations was particularly descriptive of the difference in the ability of SRB to decrease sulfates. Flow cytometry confirmed three concentrations for the study: sample 1: 8×10^4 cells/mL, sample 2: 13×10^4 cells/mL, and sample 3: 21×10^4 cells/mL. The clearance due to sulfate utilization was obtained in all three doses (Fig. 1h); although the maximum sulfate removal was recorded with sample 3 at a concentration of 21×10^4 cells/mL.

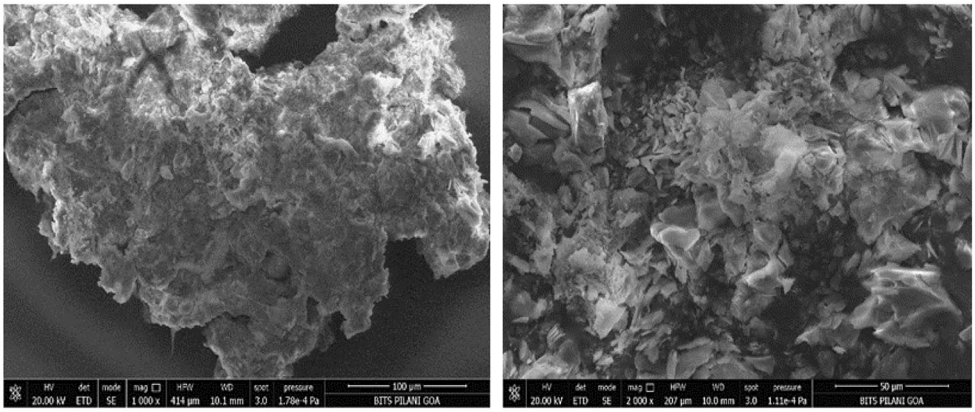

Fig. 4 SEM images of the black crust sample collected from St. Augustine facade, old-goa, India, from a black patina-covered wall, appearing unsymmetrical, with short fissures and crystals of various sizes, with non-uniform morphology; figures a and b show black crust sample examined at 100 and 50 µm scales respectively

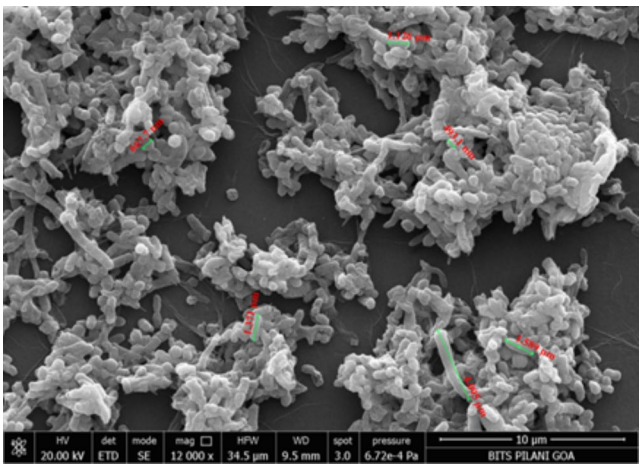

Fig. 5 SEM image indicates a consortium of *P. azotoformans* strain PgBE29 and *A. ellisii* strain LMG 26155 used to remove sulfates from the black crust from the monument surface before mixing into a carbogel carrier at 10 µm scale

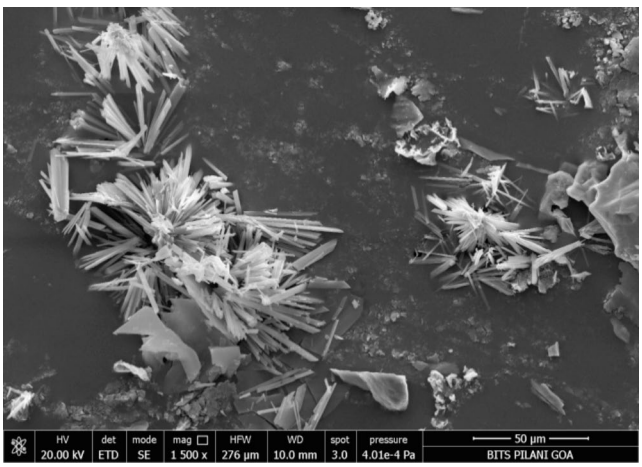

Fig. 6 SEM image of artificially produced gypsum crystals at 50 µm scale depicting needle-like structures formed on the makranta marble surface when exposed to 5 M H_2SO_4 for 1 h, followed by seven days of exposure to an open atmosphere at 50 µm scale

Increasing the number of applications to five

The results indicate a gradual rise in sulfate removal as the number of treatments increased. A sample where only a carbogel carrier was applied gives minimal clearance after three applications, confirming its inefficiency in removing pollutants from the wall alone. However, the removal of sulfates due to SRB is dominant; specifically, sulfur and its derivatives are being removed. When all three concentrations are compared, it is clear that Sample 3, with a concentration of 21×10^4 cells/mL, has the most significant potential for sulfate reduction. As a result, this cell concentration can be exploited for further research (Fig. 1k, o, m, and q). Furthermore, as the number of applications expanded from three to five, the efficiency increased, demonstrating the system's potential.

Discussion

Our results revealed the irregular morphology of the crust and the elemental composition of the damaged layers in terms of remarkable elements. Magnesium (Mg), Al, Si, C, O, calcium (Ca), Fe, and S were the most abundant elements. At the same time, Mn, K, titanium (T), Zn, Cl, phosphorus (P), and copper (Cu) were found in lower concentrations. La Russa et al. (2017) and Ruffolo et al. (2015a) reported similar elements in their investigations. However, the weight% for each element may differ. The presence of components in lesser quantities may be attributable to impure elements in the black crust (Galletti et al. 1997; Ghedini et al. 2004; Ruffolo et al. 2015, 2023; Singh and Yadav 2023). According to Comite et al. (2020), Ba-sulfate, silicates, Ca-phosphate, Pb, and Fe-rich residues, as well as Zn-containing particles, in crusts have a powdered texture that is primarily comprised of granules and hexagonal crystals of gypsum, with a small number of tabular crystals. Spherical carbon

Table 2 EDX analysis (mean ± SD, n = 3) of the biotransformation studies on marble (before and after treatment, depicts the application of sulfate-reducing bacteria for the removal of artificially produced gypsum crystals on the surface of Makranta marble.)

	After treatment			Before treatment		
Elements	Weight%	Atomic %	Net. Int.	Weight%	Atomic %	Net. Int.
C	11.133 ± 9.252	17.567 ± 13.354	10.600 ± 9.042	2.875 ± 2.345	4.900 ± 4.109	2.450 ± 3.430
O	46.400 ± 1.442	57.333 ± 3.717	48.067 ± 20.603	50.550 ± 1.088	65.250 ± 3.379	58.050 ± 19.907
Al	1.233 ± 0.737	0.900 ± 0.557	4.367 ± 2.485	0.375 ± 0.750	0.300 ± 0.600	1.425 ± 2.850
Si	9.833 ± 10.731	7.000 ± 8.088	40.600 ± 41.053	12.425 ± 12.813	8.775 ± 8.864	44.150 ± 42.942
S	0.567 ± 0.513	0.333 ± 0.306	2.333 ± 2.108	4.925 ± 6.996	3.275 ± 4.826	19.350 ± 29.922
Ca	22.333 ± 17.400	11.067 ± 9.114	62.067 ± 47.544	15.250 ± 19.312	8.075 ± 10.224	44.650 ± 61.787
Fe	2.933 ± 3.062	1.067 ± 1.172	4.400 ± 4.518	1.450 ± 1.900	0.525 ± 0.570	2.025 ± 2.585
Mg	5.533 ± 9.584	4.767 ± 8.256	17.700 ± 30.657	8.200 ± 8.669	6.675 ± 6.927	23.300 ± 24.418
K	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	4.000 ± 8.000	2.250 ± 4.500	12.600 ± 25.200

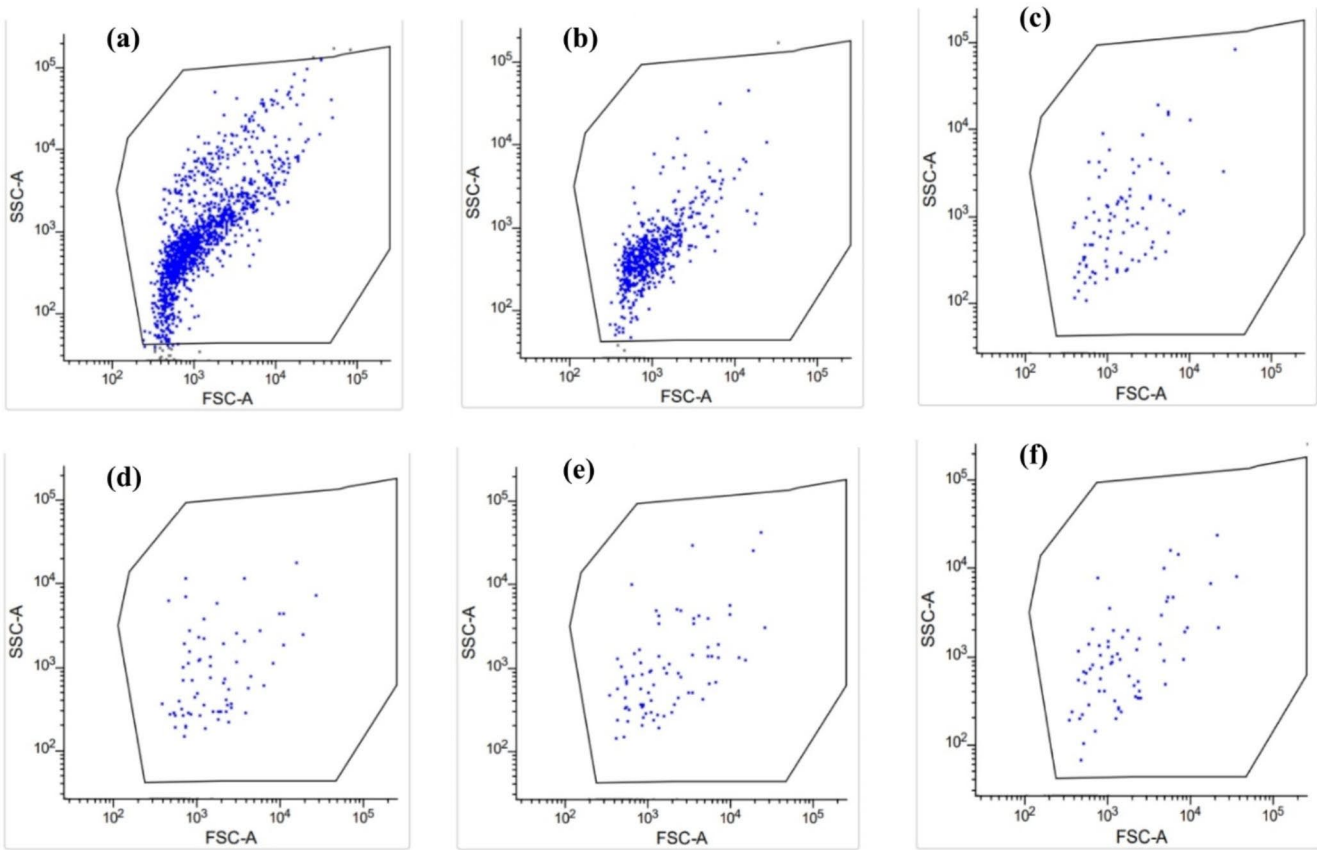

Fig. 7 Determination of cell concentration using Flow cytometry (**a**) undiluted cells of *P. azotoformans* strain PgBE29 and *A. ellisii* strain LMG 26155 (**b**) diluted cell suspension of 10^{-1} (**c**) diluted cell suspension of 10^{-2} (**d**) diluted cell suspension of 10^{-3} (**e**) diluted cell suspension of 10^{-4} (f) diluted cell suspension of 10^{-5}

and metal particles, primarily bromine (Br) and lead (Pb), in combination with Cl and K, were more frequent in crusts. EDS tests were also used to detect the accumulation of tiny metal particles (e.g., chromium and iron). The release of heavy metals Zn and Cu can be linked to the burning of fossil fuel particles, generated by friction or the wear of vehicle mechanical parts, and asphalt wear. The primary source of Zn is leaded gasoline, utilized until the end of the 1990s. The combustion particles in varying quantities in the three samples tested exhibit spherical porous morphologies and differ in diameter between 10 and 100 µm. The chemical composition of the crusts indicates the presence of S and Ca; this is due to gypsum, the primary component of the crusts.

In contrast, Al and Fe can be attributed to atmospheric particle deposits, and the existence of Si can be attributed to both atmospheric origin and particles of siloxane used in previous renovations. The presence of chloride may be

attributed to aerosol transmission from the Mandovi River, 428 m from St. Augustine Tower. The crusts comprise gypsum crystals, which can trap air particles and facilitate precipitate development (Mitsos et al. 2022). Some accessory components are pollen, soil particles, fly ash, microbes, and inorganic residues. The average concentrations of each crust component were compared to those in unaltered stones to determine the degree of composition of elements present in black crusts. Ranalli et al. (2005) reported that the effectiveness of the live microbial cell application over that of enzymes in removing sulfates and other contaminants is apparently due to the adaptability and activities of the bacteria. Bacterial cells have been shown to secrete inducible and constitutive enzymes that degrade various compounds. Inducible enzymes can only be synthesized in the presence of a substrate, resulting in a regulatory effect (Ruginescu et al. 2022). As a result, using microorganisms is more successful than using a single enzyme that disintegrates specific linkages. Furthermore, the constitutive and induced enzymes of live cells of diverse bacteria like *Pseudomonas sp.* can break up numerous organic complex chemical bonds (Ranalli et al. 2005).

Water is used by *Pseudomonas sp.* that secrete hydrolytic enzymes (such as lipases, peroxidases, proteases, cellulases, esterases, nitrilases, cutinases, and amylases) to break chemical bonds and break down larger molecules into smaller ones, decreasing the toxicity of pollutants. They facilitate the water-mediated cleavage of C-C, S-P, C-N, S-S, C-O, S-N, and other bonds and catalyze a number of related processes, including condensations and alcoholic reactions (Bhandari et al. 2021). SRB convert sulfate to gaseous hydrogen sulfide (Hedlund 2023). Microbial hydrolytic activity can also remove organic deposits from artworks, such as lipidic, carbohydrate, and proteinacious residues (Soffritti et al. 2019; Guimaraes 2012). Some metabolic activities of bacteria cause calcite precipitation via active and passive processes (Baidya et al. 2023). Other sulfate-reduction approaches include assimilatory and dissimilatory pathways. In the assimilatory process, sulfate is reduced to sulfide and then transformed into cysteine.

In contrast, sulfate is employed as a terminal electron acceptor of the respiratory process in the dissimilatory pathway, producing vast amounts of sulfide. SRB were used in this study to reduce the sulfates and thereby eliminate the crust at the field and lab ales. The current work focuses on the bioapplication of the *P. azotoformans* strain PgBE29 and *A. ellisii* strain LMG 26155 consortium mixed in a carbogel carrier to remove sulfates from the black crust on a lab scale and in onsite studies. The method of application is determined by the type of modification, the art medium, the treatment sites, and the metabolic activity of the selected bacteria (aerobic and anaerobic). Based on the analysis, we suggest not exceeding the number of applications beyond three to increase the performance. The bioremediation by carbogel carrier without bacterial cells is negligible in the first three applications, while there is an enhanced contribution by carbogel itself after three applications. Bacterial treatment in artwork bio-remediation necessitates the application of selected microbes to the artwork surface via spray, brush, or compress. Determination of cell concentration using flow cytometry analysis would be an added advantage. According to our understanding, this is the first study to use facultative anaerobic bacteria in consortia applied as jelly with a brush to remove sulfates off monument surfaces in Goa. Exploiting microbes for their potential, which is naturally present in an environment, is an alternative green technology for conservators. However, results were encountered even at lower concentrations of cells, which may be because of the ideal interaction of the two strains. The most significant advantage of microbial/enzymatic bio restoration over traditional approaches (chemical, physical, and mechanical) is that it is non-detrimental, removing only external or changed components from the altered surface.

Furthermore, microbial cells have non-specific action, whereas the use of enzymes is highly selective and limited. Another benefit is the employment of safe microorganisms for operators and the environment. Finally, a cost-benefit analysis demonstrated that this technological innovation could be used in domains other than cultural heritage. Depending on the results from studies on St. Augustine Tower, we can use advanced technologies based on different strains of microorganisms already used to remove the black crust when planning the following experiments. In other studies, bio restoration studies reveal the ability of SRB, *D. vulgaris*, to successfully remove sulfates from the crust on the marble surface (Cappitelli et al. 2006, 2007; De Gusseme et al. 2009; Ranalli et al. 1997; Webster and May 2006; Ruffolo et al. 2023). *Pseudomonas sp.* is also used to remove undesirable compounds from weathered stone surfaces and environments (Alfano et al. 2011; Toerien et al. 1968; Potysz and Bartz 2023). *Arcobacter sp.* uses sulfur and nitrates as electron acceptors and thus can grow on elemental sulfur thiosulfates, including complex organic substrates (De Gusseme et al. 2009; Roalkvam et al. 2015). Using a consortium of *P. azotoformans* strain PgBE29 and *A. ellisii* strain LMG 26155 isolated from the digestate of a biogas plant proved beneficial concerning the sulfate removal efficiency exhibited. The SRB can remove sulfates from the wastewater before the anaerobic treatment during the biogas production (van den Brand et al. 2018).

As a result, the digestate from the biogas plant is a good source for isolating sulfate-reducing bacteria for exploitation in biorestoration studies (Suyasa et al. 2023; Diao et al. 2023). In the work of Gauri et al. (1992), the sulfated

stone was immersed in a medium containing cells of standard cultures of *D. desulfuricans* and *D. vulgaris*, exhibiting a black crust removal efficiency of 40–100% after 60–84 h of treatment. However, this technique is not feasible for more prominent monuments and will damage the layers beneath the black crust due to immersion (Cappitelli et al. 2006). A more practical approach is presented when organic or inorganic carriers are used that can be easily removed from the monument surfaces. In the studies of Ranalli et al. (1996) and Ranalli et al. (1997), the carrier used was sepiolite colonized with cells of *D. desulfuricans* and *D. vulgaris*, showing 80% of sulfate removal. Nitrates were also removed using strains of *Pseudomonas stutzeri* in an anaerobic environment (Tomić et al. 2022). Yet, it exhibited the issue of iron precipitation leading to blackening. Also, it did not give evidence of the amount of water available to the cells for metabolic activities after colonization. Therefore, in the study of Cappitelli et al. (2006) and Cappitelli et al. (2007), organic carriers like carbogel were used, showing 98% sulfate removal. The carbogel was feasible as it forms a compact homogenous, system and is thus easy to handle. An added advantage is that it has a high capacity for retaining water, with consequential evanescence over the long run (Bosch-Roig et al. 2015). After the treatment, the inactivity of cells inoculated into the medium suggests no requirement to remove the cells from the monument surface using a unique technique.

In the field investigation in Milan, Italy, *D. vulgaris ATCC 29,579* was embedded in a carbogel carrier. The culture growth medium had been altered by eliminating iron to prevent precipitation. Employing SRB to remove black crust has succeeded in sulfate removal using the method used at Milan Cathedral (Ranalli et al. 1997; Webster and May 2006; Cappitelli et al. 2006). Many researchers have proved the efficiency of SRB in removing sulfates from the black crust, yet the time taken for each varies from 2 to 30 days (Webster and May 2006; Tiano et al. 2006). We focus on the treatment time, which is between 15 and 45 h. Laser cleaning is a pertinent offering in the removal of stone alterations. However, using lasers is costly. In some cases, they do not entirely remove the deposits and cause a change in the color of the treated area (Ramírez et al. 2005; Junier and Joseph 2017). Biorestoration of stoneworks using microbes and their enzymes has also been reported by Bellucci et al. (1999) and Ranalli and Zanardini (2021). In the work of Gioventù and Lorenzi (2013), a comparison was made between three different cleaning methods: bio-cleaning using *D. vulgaris* trapped in carbogel, laser cleaning, and chemical cleaning. Still, a longer treatment time was required, along with many applications. Also, this study did not show the effect of only carbogel without bacterial cells on the black crust serving as a control. Many studies also failed to include architectural evidence of the site chosen for the experiment, making it challenging to understand where the experiment was conducted. This study focuses on architectural documentation documenting the overall survey, the measured drawing of the stone facade, and the mapping of discolored areas, a pre-requisite for conserving heritage as per the International Charter of Conservation. Photographic documentation enabled the preparation of measured drawings and the mapping of concerned decay mechanisms. It also allowed us to compare areas under control and experiment with varying samples. We must note that the analysis of the selected areas in our study showed that black sulfated crust consists of distinct components in various other regions of the same church wall. Therefore, it is of utmost importance to recall that the differences in results could be because of the heterogeneous distribution of compounds present. Despite the diversity of techniques used for the restoration of heritage sites, one reason for researching newer technologies is to devise a method that is feasible for the removal of thin as well as thick layers of black crust with excellent efficiency, allowing the process to stop at any specific level (Eskici and Kabaoglu 2010; Sabatini et al. 2000). In our study, SRB treatment fulfilled this demand. However, the several applications may require treating thick crust on the monument. The results confirm the efficacy of SRB in removing black crust; however, the practicability of this method has to be estimated by further studies in terms of the application number and the treatment time. This can be assessed by considering components such as thickness and elements present in the crust (Cappitelli et al. 2007). The uniformity of the crust is another critical factor that depends upon the climate, location, type of material, and air pollution.

Conclusion

To conclude, extensive research reports have demonstrated the promise of innovative microbiological systems utilizing microorganisms to combat decay and efficiently remove pollutants, offering potential alternatives for cultural heritage restoration efforts. The current study isolated facultative anaerobic strains, *P. azotoformans* strain PgBE29 and *A. ellisii* strain LMG 26155, which exhibited sulfate-reducing ability from the black crust when used as consortium. Our observations suggest that exploration of these microbial strains has shown significant results in addressing a broad spectrum of undesirable substances commonly found on the surfaces of historical monuments. This study helped to establish a more comprehensive understanding of the potential of these strains to address the impact of pollutants on historical monuments and offered valuable insights for

the future. It also provides a sustainable solution and eco-friendly approach for cultural heritage restoration methods in future applications.

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Declarations

Competing interests the authors declare that they have no competing interests.

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