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RESEARCH

Influence of bacterial biopolymers on physical properties of experimental limestone blocks

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Abstract

This article describes the consolidation effects of bacterial biopolymers synthesized by biofilm bacteria colonizing Mayan limestone buildings on the surface properties of limestone blocks, including disaggregation, hardness, and total color change at the laboratory level. The biopolymers evaluated, produced by bacterial isolates TM1B-488, TM1B-489, TM1B-349, and TM1B-464, influenced surface properties at different levels. 16S rRNA gene sequences analysis showed that isolate TM1B-349 was related with *Psychrobacter* sp. strain Marseille P-5312, TM1B-464 was related with *Agrococcus terreus* strain BT116, and isolates TM1B-488 and TM1B-489 were related with *Xanthomonas citri* pv. mangiferaeindicae strain XC01. Biopolymer A reduced the surface disaggregation of the material (26%) compared to the untreated control, as revealed by the peeling test, followed by biopolymer B (10%), while the remaining biopolymers had a negligible effect. The cactus biopolymer reduced disaggregation at higher levels (37%). On the other hand, there was a similar concomitant increase in surface hardness of limestone samples coated with biopolymer A (34%) and biopolymer B (32%), higher than biopolymers C (10%) and D (19%). Total color change for all treatments was below the threshold value of 5, indicating a non-significant color alteration. Partial chemical characterization of best-performing biopolymer (A) suggests its probable glycoprotein nature, whose constitutive acidic monosaccharides probably contributed to higher adherence to the limestone surfaces, contributing to surface stabilization, hardening the surface, and decreasing surface decohesion. These preliminary findings suggest its potential application in bioconsolidants, but further studies are required.

Keywords Bacterial biopolymers · Bioconsolidation · Historical monuments · Limestone · Physical properties · 16S rRNA

Introduction

The stone used for cultural heritage and modern buildings is weathered from the moment it is quarried and exposed to the atmosphere by various natural processes involving physical, chemical, and biological agents, which operate simultaneously. Human conservation practices also induce deterioration due to insufficient maintenance or inappropriate restoration activities (Fitzner and Heinrichs 2002). Carbonate rocks such as limestone and marble are particularly prone to weathering and are among the most common stones used to build and restore monuments (Rodriguez-Navarro et al. 2003). Mayan sites in southern Mexico and Central America are exposed to high temperatures and relative humidity, leading to increased deterioration. The Mayans mainly used soft limestone to construct their buildings. The challenging climatic conditions of the tropical forest of this region favor

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microbial biofilm growth that leads to the production of deteriorating metabolites such as organic and inorganic acids and extracellular polymeric substances (Ortega-Morales et al. 2000; Scheerer et al. 2009; Gaylarde and Little 2022). The relative mechanical weakness of limestone then allows the direct dissolution of the structure.

Extracellular polymeric substances (EPS) are often cited as potential mediators of biodeterioration by directly and indirectly acting as bioadhesins, trapping dirt and other particulate materials, increasing the disfiguring effects of the biofilm, and making the structure more difficult to clean and restore (Scheerer et al. 2009). However, EPS can also mediate important positive processes such as biosorption and biomineralization, modifying the local surface biogeochemical environment and contributing to the stability of surfaces, and acting as barriers between the stone and the atmosphere (Ortega-Morales et al. 2001; Elert et al. 2021). This is consistent with the recent view that microorganisms can also lead to the protection of stone (Pina 2014; Favero-Long and Viles 2020). This highlights the complex nature of the interaction between microbes and minerals. Indeed, an emergent avenue of research is associated with biopreservation, defined as the application of consolidation, cleaning, and active or passive protection treatments induced or mediated by microorganisms (Ortega-Morales and Gaylarde 2021). Biopreservation includes the *in-situ* consolidation of ornamental materials through microorganism-induced biomineralization and a passive interaction between EPS and stone (Rodriguez-Navarro et al. 2003; Decho 2010). Although biotechnology has been recently recognized as an emerging field of high relevance in heritage science, few restorers are aware of the potential that biotechnological approaches can provide in their conservation tasks. Indeed, biotechnology can aid in understanding and improving the properties of building materials in an ecologically sustainable manner that is acceptable to the vision of the restorer community, who are particularly unwilling to use synthetic materials.

Consolidation of stone requires that the materials applied should effectively protect and by compatible with stone surfaces. Substratum integrity, mainly determined by high surface hardness and low susceptibility to flaking, exfoliating and granular disaggregation, are key variables associated with assessing the effectiveness of consolidation treatments. Among other properties, consolidants should increase surface hardness and reduce disaggregation of stone substrata (Delgado-Rodrigues and Grossi 2007; De Rosario et al. 2015). The consolidant should also not interfere with the appearance of the surface, especially in terms of its color, which bears aesthetic value (Ferreira-Pinto and Delgado-Rodrigues 2012; Becherini et al. 2018).

The current article reports experimental laboratory results of treating limestone specimens with biopolymers derived from epilithic bacteria isolated from ancient Mayan monuments. It is hypothesized that biopolymers may act as barriers, inducing changes in surface hardness and disaggregation as indicators of surface consolidation but not substantially altering the color. The biopolymer that performed best was partially characterized.

Materials and methods

Characterization of rock coupons

High porosity limestone samples, composed mainly of calcium carbonate, denominated RP (rocaporosa = porous rock), were used in this study. They were provided by Artesanías en Mármol y Canteras de Yucatán S. C. de R. L. from the state of Yucatan, Mexico and characterized in the Department of Applied Physics, CINVESTAV-Mérida, Mexico (Table 1). High porosity rock in the Yucatan Peninsula is a fossiliferous rock of high porosity and variable hardness, found between 5 and 9 m in depth, with various cream shades, and encrusted with marine debris. High porosity rock was used as it is more bioreceptive because it holds higher amounts of water for sustained microbial growth (Gómez -Cornelio et al. 2016).

Table 1 shows the characteristics of the chosen limestone material. Slabs were cut into blocks of 0.05 m × 0.05 m × 0.01 m (Carmona-Quiroga et al. 2017).

Isolation, screening, identification and EPS production

Microorganisms were isolated from epilithic biofilms following the protocol described by Gaylarde et al. (2012). Samples were taken from archaeological sites of Chicanná, located south of Campeche, Mexico (Fig. 1). Microorganisms were isolated by the serial dilution spread-plate technique. Dilutions of 1×10^{-2} to 1×10^{-4} in physiological saline solution (NaCl 0.85% w/v), were inoculated into various culture media to maximize recovery of microorganisms; these were *Bacillus* agar (Neogen), Nutrient

Table 1 Characteristics of the limestone specimens for the study

Physical property	Value
Water absorption by weight	1.9%
Water absorption by volume	4.5%
Density	2338.7 kg m^{-3}
Porosity	6%
Resistance to pressure	140.19 kg cm^{-2}

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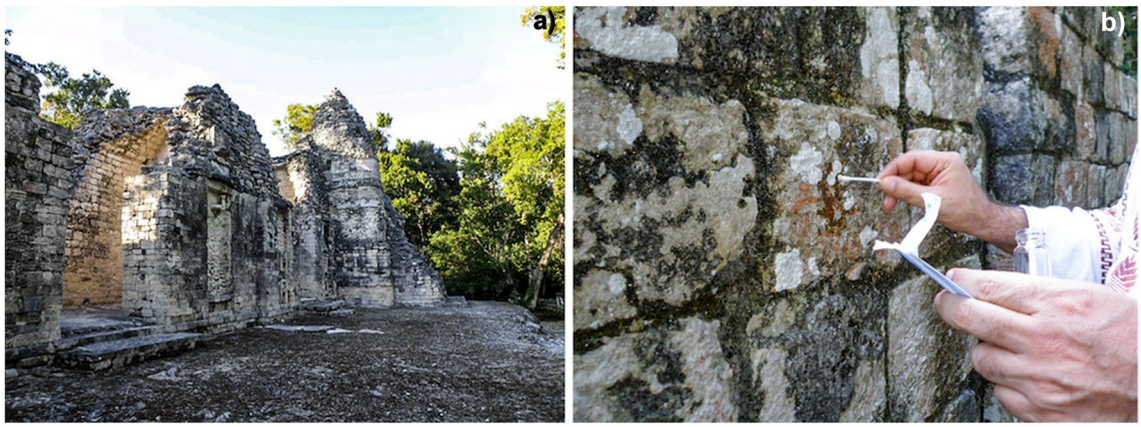

Fig. 1 **a** Structure I at Chicanná exhibiting colonization of a range of biofilms. **b** Aseptic swabbing of biofilms for bacterial isolation from a stone surface

agar (BD Difco- Becton Dickinson and Company), Potato Dextrose Agar (BD Difco-Becton Dickinson Company), R2A (BD Difco-Becton Dickinson Company), Tryptone Soy Agar (TSA), and Actinomycete Isolation Agar (Himedia). All plates were cultured at 28 °C for 1–7 days and checked daily to isolate representative colonies of each visual type. Isolates were then successively restreaked for purification, checked by microscopy. All bacterial isolates were preserved as glycerol suspensions (20%, w/v) at − 80 °C and deposited in the culture collection of the Center for Research in Environmental Microbiology and Biotechnology, Autonomous University of Campeche.

A total of 203 epilithic bacterial isolates were recovered from the archaeological site during this sampling survey. These were cultured for 48 h at 28 °C in Tryptone Soy Agar (BD Bioxon) supplemented with glucose (1% w/v) (Jalmek®), (TSA/gluc). Bacterial isolates were screened, and those with a mucous (slimy) appearance, indicative of EPS production, were selected. The presumptive EPS-producing isolates were reinoculated on TSA/gluc plates. Colonies were scraped from the agar surface with a spatula and transferred to tubes of saline solution (NaCl 0.85% w/v), The polysaccharide content was assayed using the method of Dubois et al. (1956); further assessment of selected isolates was performed to determine the ability to produce EPS in 1 L TSB/gluc stirred at 200 rpm for 48 h at 28 °C. The EPS were extracted according to the protocol described by Camacho-Chab et al. (2013). For comparative analysis, cactus biopolymer (*Opuntia ficus-indica*) was chosen as a reference biomaterial, and the extraction was performed following the protocol described by Dominguez-Canales et al. (2011). Four bacterial isolates (TM1B-488, TM1B-489, TM1B-349, and TM1B-464) and their corresponding biopolymers A, B, C, and D, were retained to study their influence on limestone physical properties along with identification.

16S rRNA gene sequencing of selected bacterial isolates

Bacterial isolates TM1B-488, TM1B-489, TM1B-349, and TM1B-464 were grown for extraction of genomic DNA using the Quick DNA Fecal/Soil Microbe Miniprep kit (Zymo Research, Irvine, CA, USA) following the manufacturer´s instructions. The DNA concentration was determined with the QuantiFluor® dsDNA kit (Promega Corporation, Madison, WI, USA) in a Quantus Fluorometer according to the manufacturer's protocol. DNA integrity was verified on a 1% agarose gel. The primers 8F (5′-AGAGTTTGATCMTGGC-3′) and 1492R (5′-TACCTTGTTACGACTT-3′) were used to amplify 16S rRNA genes as previously described (Buchholz-Cleven et al. 1997). Also, primers 787R (5′-GGACTACCAGGGTATCTAAT-3′) and 518F (5′-CCAGCAGCCGCGGTAAT-3′) were used for sequencing in the Biotechnology Institute of the National Autonomous University of Mexico. Almost complete sequences of 1400–1500 base pairs were obtained manually editing each sequence using Chromas 2.6.6 (Technelysium Pty Ltd, South Brisbane, AU) and assembled with BioEdit 7.2.5 (Hall 1999). BLASTN was used to compare the sequences with the nucleotide database (Zhang et al. 2000).

Preparation of the phylogenetic tree

Consensus sequences were taken and a BLASTn search was performed. Subsequently, the consensus sequences and the BLAST results were aligned in the MEGA program (Version 10), with the ClustalW algorithm with a Clustal substitution matrix. The alignment was used to determine the best evolutionary model for phylogenetic reconstruction using the "Find Best DNA/Protein Models" algorithm within MEGA.

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The phylogeny tree was developed using Maximum Likelihood (ML) with the 3-parameter Tamura model with Gamma distribution and Bootstrap value of 200 repetitions.

Coating of stone specimens with biopolymers

A solution of each biopolymer was prepared at a concentration of 1% (w/v) in absolute alcohol. The treated limestone samples were stored in an environmental chamber at 30 °C and relative humidity (RH) of 70 ± 88%. Each biopolymer solution was applied to the limestone specimens using a small brush. The surface of each stone specimen was covered ten times, waiting approximately 2 min between each application. Four different biopolymer treatments (A, B, C, and D), a control (non-treated stone), and a reference biomaterial (cactus biopolymer) were tested. Each treatment was applied in triplicate for each trial. The physical properties of the treated and untreated specimens were determined after 30 days of storage in the environmental chamber, as indicated below.

Evaluation of the properties of rock samples

The physical properties of (i) surface hardness and (ii) disaggregation (surface peeling) were evaluated on the rock specimens as indicators of biopolymer effectiveness. Color change (iii) was measured to determine compatibility. A consolidation effect is reflected by a change of these properties, an increase in stone surfaces concomitant with lowered surface disaggregation; compatibility was assessed according to a total color change threshold value below 5 for all treatments, this figure indicating a non-significant color alteration (Prieto et al. 2010; López-Arce et al. 2013; Camacho-Chab et al. 2021). Cactus biopolymer was included as a reference biomaterial since it has been shown to have porous material consolidation properties (Kita 2013; Pérez et al. 2015).

Surface disaggregation (peeling test)

The amount of material peeled from the surface by Scotch Tape can be linked to the loss of cohesive surface properties. Single-sided transparent (Scotch 550 3 M®) tape, 24 mm wide, was used. The transparent adhesive tape was cut into 50 mm length pieces to give a contact surface of 12 cm^2 (2.4 × 5 cm). Ten pieces were weighed, and the average was used as the initial weight for the peel test analysis. The tape was applied to the surface of the rock specimens by hand, using standard pressure to ensure homogeneous adhesion to the analyzed surface. After approximately 90 s, the tape was removed by grasping the free end and pulling it steadily (not abruptly) at a speed of approximately 10 mm s^{-1} and an angle of 90°. Subsequently, the tape was weighed to determine the weight of the attached material removed from the limestone surface. This process was repeated ten times over the same area to determine the cohesion of the rock surfaces (Drdácký et al. 2012).

Surface hardness

Surface hardness was evaluated with a portable hardness tester (Equotip 3) using a modification of the single impact method established by Viles et al. (2011). Twenty measurements were made at random locations on each of the blocks. The average maximum Leeb hardness value (HLmax) was calculated for each surface for the six highest readings. The data were expressed on the "Leeb hardness" scale (1–999), using the following formula:

$$\mathrm{HLmax} = \mathrm{Vr/Vi} \times 1000$$

where Vr = bounce speed and Vi = impact speed, as measured by Equotip 3.

Color change after biopolymer application

To determine the color change induced by the treatments, the total color change ($\Delta E \times ab$) was determined by reading the color parameter before, immediately after, and 30 days after biopolymer application.

The total color change was determined on five random 5 cm × 5 cm areas of each coupon using a portable spectrophotometer (Konica Minolta cm-700D) to obtain the CIELAB coordinates. The parametric conditions used were: illuminant D65, observer 2°, and 10-mm diameter viewing (Sanmartín et al. 2012). The CIEL*a*b* coordinates were transformed into RGB channels to obtain a visual representation of the color (el Halim et al. 2022). The ΔL*, Δa*, and Δb* values needed to calculate the total color change were calculated as described by Sanmartín et al. 2012 and Camacho-Chab et al. 2021.

Finally, using the $\Delta E \times ab$ formula proposed by La Comisión Internacional de l'Echairage 1976-L*a*b* (CIE 1976), the total color change was calculated for each surface treated with the biopolymer.

$$\Delta E \times ab = \left[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2\right]^{1/2}$$

where $\Delta E \times ab$ represents the total color difference of each coupon after biopolymer treatment.

Statistical analysis

Data were analyzed using independent t-student parametric tests to compare differences in intra-mean groups of each of the treatments and evidence of the statistically significant

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difference in the color change caused by the coating over time. A U-Mann–Whitney test was used for variables that did not meet the assumptions of normality and homogeneity of variances. Two-way ANOVA variance analyses and the Tukey post hoc test were performed to compare the intergroup statistical difference, to determine the treatment with the least surface color alteration. These latter tests were performed with the total color change test ΔE. The statistical analyses were performed with the jamovi 2.3.13.0 statistical program, and the differences were considered statistically significant when obtaining a value of $p \leq 0.05$.

Chemical characterization of best performing bacterial biopolymer

The best performing biopolymer (based on the assessment of physical properties) was characterized to determine its carbohydrate (Dubois et al. (1956) and protein content (Lowry et al. 1951), using glucose (Sigma®) and albumin (Sigma®), relatively, as standards. In addition, a partial analysis of monosaccharide content was carried out as described below. The biopolymer was hydrolyzed by modifying the protocol described by Lutfia et al. (2020). It was dissolved in distilled water (5 mg mL^{-1}) and 250 µL were mixed with 1 mL of 4 M trifluoroacetic acid. The solution was heated at 120 °C for 2 h in sealed glass tubes and passed through a 0.22 µm pore filter. Finally, samples were dried under a nitrogen stream. Monosaccharide composition was analyzed by HPLC (Agilent Infinity 1260) coupled with a refractive index detector (RID) and a ligand exchange column (Agilent Hi-Plex H, 300 × 7.7 mm) at 35 °C. The mobile phase was 0.05 M sulfuric acid (H_2SO_4) with a flow rate of 0.6 mL min^{-1}. The sample was dissolved in HPLC water, and the injection volume was 20 µL. Standards used for peak identification were: D-(+)-galactose, D-(+)-galacturonic acid, D-glucuronic acid, D-(+)-xylose, L-(+)-arabinose, D-(+)-glucose, D-(+)-mannose and L-rhamnose (all Sigma® standards). The biopolymer produced by the prickly pear cactus was also analyzed.

Results

Characterization of selected epilithic bacterial biopolymers

Four isolates (TM1B-488, TM1B-489, TM1B-349 and TM1B-464) were selected for determination of the biopolymer carbohydrate content (Table 2), since they exhibited confluent mucoid morphotypes and produced their corresponding biopolymers at varying levels. TSBgluc growth allowed various production yields. The highest EPS production was recorded for isolate TM1- 489 (4.23 g L^{-1}), and the lowest for isolate TMIB-349 (0.022 g L^{-1}). The highest carbohydrate content (31 ± 0.003%) was associated with biopolymer A and the lowest with biopolymer C (17 ± 0.03%). The cactus biopolymer had a higher carbohydrate proportion than all bacterial biopolymers assessed.

Table 2 Carbohydrate content and production level of biopolymers in liquid TSB of selected epilithic biofilm bacteria

Biopolymer designation	Bacterial isolate	Carbohydrate content (%)	Yield (g L^{-1})
Cactus biopolymer	N.A[a]	39 ± 0.02	N.A
Biopolymer A	TM1B-488	31 ± 0.003	3.50 g L^{-1}
Biopolymer B	TM1B-489	27 ± 0.04	4.23 g L^{-1}
Biopolymer C	TM1B-349	17 ± 0.03	0.22 g L^{-1}
Biopolymer D	TM1B-464	25 ± 0.02	0.80 g L^{-1}

[a]Not Applicable

Sequence analysis of the 16S rRNA gene of the selected epilytic isolates

BLASTN analysis of almost complete 16S rRNA gene sequences showed that isolate TM1B-349 had 99.79% identity with *Psychrobacter* sp. strain Marseille P-5312 (GeneBank accession number LT985455.1); TM1B-464 had 99.79% identity with *Agrococcus terreus* strain BT116 (GeneBank accession number MH934923.1); isolates TM1B-488 and TM1B-489 had 99.58% and 99.86% identity respectively with *Xanthomonas citri* pv. mangiferaeindicae strain XC01 (GeneBank accession number CP016836.1). Interestingly, both isolates identified as *Xanthomonas citri* produced biopolymers that reduced the surface disaggregation of the material in comparison to the untreated control, while biopolymers from isolates of the genera *Psychrobacter* and *Agrococcus* showed a negligible effect according to the peeling test. Figure 2, shows and confirms the taxonomic assignation in the reconstruction phylogenetic tree.

Influence of EPS on physical properties of limestone

Limestone surface cohesion

Deteriorating agents induce a range of disaggregation patterns of limestone grains. This reduction of surface integrity leads to an increase in material removed from the surface by adhesive tape. The peeling test (Fig. 3) showed that of the four biopolymers evaluated, biopolymers B, C, and A caused a decrease in rock material removed from the surface by the adhesive tape. Biopolymer A showed the lowest detachment values, similar to the cactus biopolymer, which had a greater substrate affinity. Biopolymer D allowed the greatest

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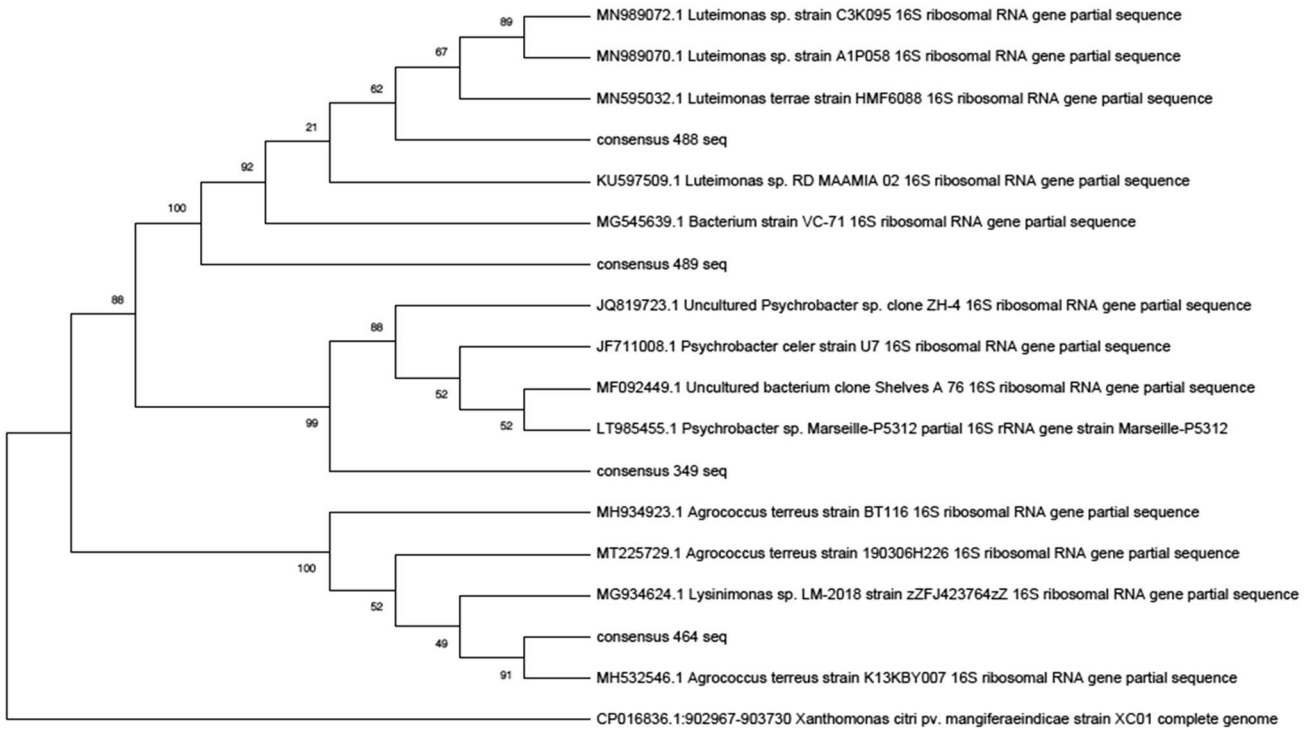

Fig. 2 Phylogenetic tree (ML). The taxonomic assignation by 16SrRNA is confirmed

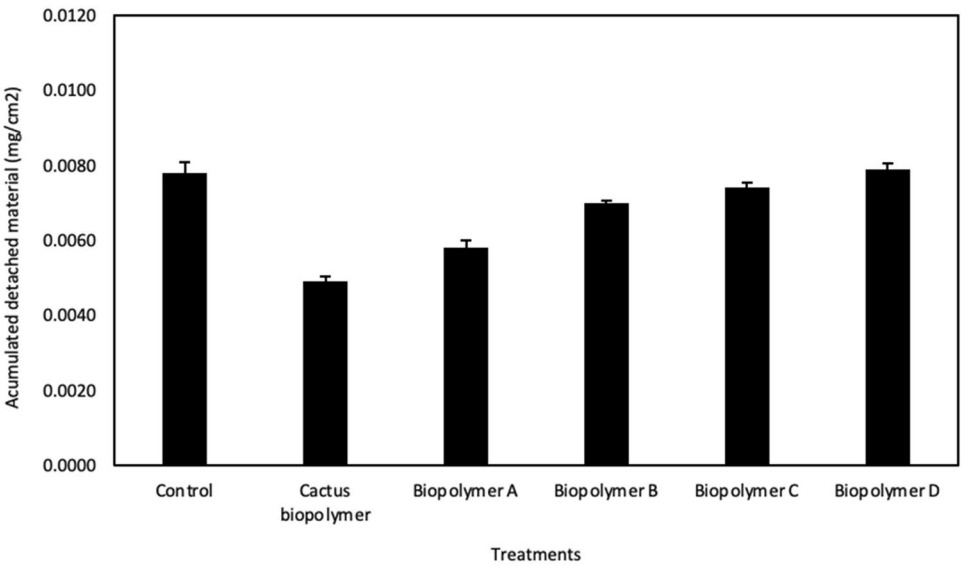

Fig. 3 Resistance to surface disaggregation. Amount of disaggregated (peeled) material onto the adhesive tape 30 days after application of various treatments: Control (uncoated sound limestone specimens), cactus biopolymer (reference biomaterial), biopolymer A, biopolymer B, biopolymer C, and biopolymer D. Values are the average of triplicate measurements; bars represent standard deviation

material removal, similar to the negative control (uncoated fresh rock specimens).

Surface hardness

The surface hardness values obtained all show an increase over the negative (untreated) control (Table 3). The positive control (cactus biopolymer) was the highest, followed by biopolymers A and B. The HL values can be interpreted as

Table 3 Surface hardness of biopolymer-coated limestone

Treatment	Leeb hardness (HL)
Control	406.7 ± 27.2
Cactus biopolymer	713.5 ± 8.3
Biopolymer A	546.7 ± 16.6
Biopolymer B	537.8 ± 18.1
Biopolymer C	447.8 ± 24
Biopolymer D	488 ± 22.1

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Table 4 CIEL*a*b* coordinates of rock surfaces before, after, and 30 days after applying biopolymer

Treatment	Time	L*	a*	b*	Color[b]
Control	Before[a]	83.95 ±1.93	3.93 ±0.60	14.85 ±2.24	
	After	85.04 ±1.46	3.14 ±0.46	13.24 ±1.26	
	30 days	85.39 ±2.25	3.27 ±0.84	12.40 ±2.34	
Cactus biopolymer	Before	83.95 ±1.93	3.93 ±0.60	14.85 ±2.24	
	After	82.40 ±1.63	3.43 ±0.49	15.92 ±1.14	
	30 days	82.38 ±1.42	3.95 ±0.79	15.60 ±0.90	
Biopolymer A	Before	83.95 ±1.93	3.93 ±0.60	14.85 ±2.24	
	After	83.45 ±2.36	2.79 ±0.17	13.05 ±0.98	
	30 days	85.40 ±0.48	2.57 ±0.14	12.61 ±1.16	
Biopolymer B	Before	83.95 ±1.93	3.93 ±0.60	14.85 ±2.24	
	After	84.80 ±0.32	3.00 ±0.21	13.62 ±0.42	
	30 days	85.27 ±1.18	2.95 ±0.48	12.98 ±1.42	
Biopolymer C	Before	83.95 ±1.93	3.93 ±0.60	14.85 ±2.24	
	After	83.23 ±1.48	3.81 ±0.50	14.57 ±0.92	
	30 days	83.97 ±1.73	3.62 ±0.22	14.15 ±0.78	
Biopolymer D	Before	83.95 ±1.93	3.93 ±0.60	14.85 ±2.24	
	After	83.46 ±1.62	3.94 ±0.36	14.76 ±1.82	
	30 days	85.41 ±1.22	3.45 ±0.37	13.90 ±2.05	

[a]Initial readings taken on limestone samples without any treatment

[b]The color of the boxes represents the visual interpretation of the Cartesian coordinates of the CIEL*a*b* color space (Camacho-Chab et al. 2021)

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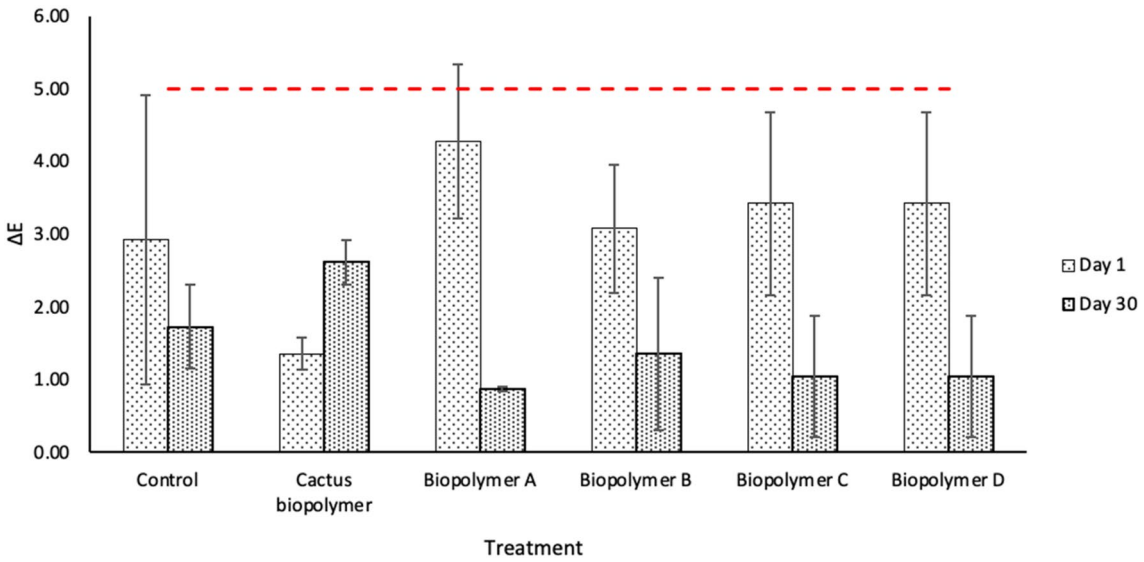

Fig. 4 Average ΔE values (three determinations) obtained before and 30 days after applying the biopolymer. The error bars represent the standard deviation. The red line represents the limit of permissible ΔE values not to consider an alteration of the surface color

Table 5 Independent t-student test results for ΔE values

Treatment	ΔE (*p*-values)	Mean difference
Control	0.371	1.20
Cactus biopolymer	0.333	− 1.26
Biopolymer A	0.023	3.41
Biopolymer B	0.094	1.73
Biopolymer C	0.051	2.39
Biopolymer D	0.051	2.39

the cohesion values of the rock structure. Together with the positive control, biopolymers A and B produced the highest values with lower standard deviations, indicating that they had managed to fill more pore spaces. The high standard deviations indicate that some parts of the highly variable stone structure are heterogeneous, with the consolidation treatment not able to seal all pores.

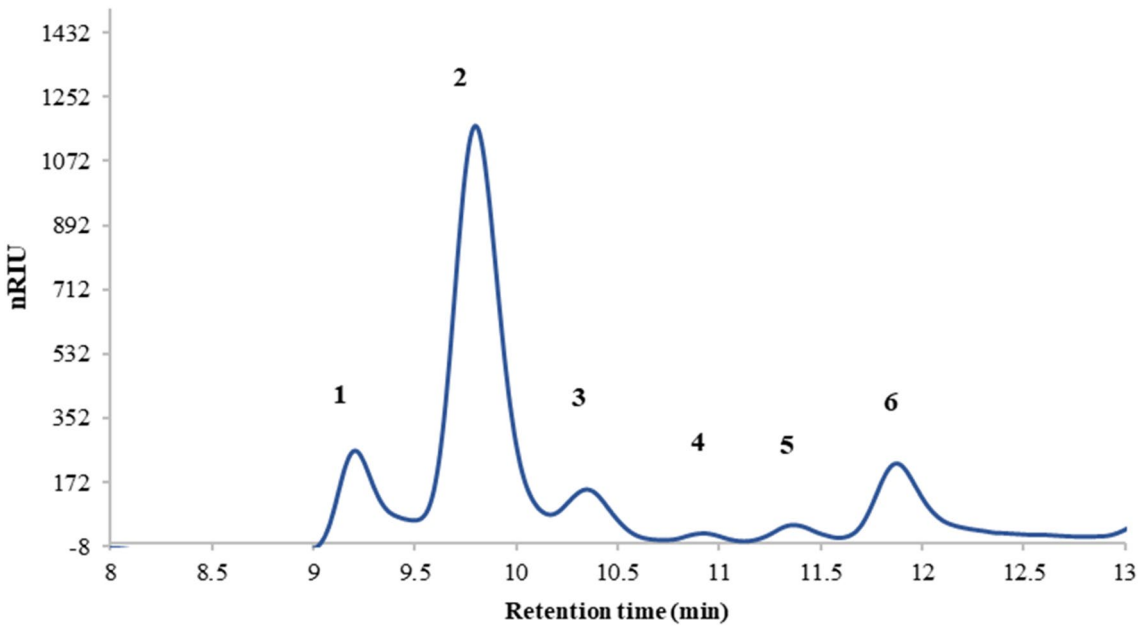

Fig. 5 Monosaccharide chromatographic profile of biopolymer A. Components are: 1. Glucuronic acid. 2. Galacturonic acid. 3. Xylose, galactose, or mannose. 4. Rhamnose. 5. Unidentified peak. 6. Arabinose

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Table 6 Monosaccharide composition of biopolymer A obtained from epilithic bacterial isolated TM1B-488 from Mayan site Chicanná, Campeche, México

Monosaccharide	Proportion (%)
Galacturonic acid	64.1
Glucuronic acid	11.8
Arabinose	13.4
Rhamnose	1
Xylose/galactose/mannose	5.6
Unidentified sugar	4.1

Color change of biopolymer-treated limestone

The estimation of the CIEL*a*b* coordinates showed a slight change in color straight after applying the different treatments (biopolymers A–D and cactus biopolymer) and 30 days after application (Table 4).

The total color difference showed that after applying the biopolymers, the color values were variable. The Cactus biopolymer-treated samples exhibited the lowest value ($\Delta E = 1.35 \pm 0.22$ SD), followed by the control with ΔE =2.93 ($\pm$ 1.99 SD), biopolymer B ($\Delta E = 3.08 \pm 0.88$ SD), biopolymer C and D (both $\Delta E = 3.43 \pm 1.26$ SD), and biopolymer A ($\Delta E = 4.28 \pm 1.06$ SD). Thirty days after application, the total color change significantly decreased in all the treatments. The cactus biopolymer-treated samples had the highest ΔE of 2.61 (± 0.31 SD) while biopolymer A had the lowest (0.87 ± 0.03 SD), as shown in Fig. 4.

Biopolymer A produced a statistically significant change in ΔE over the 30 days. Of the other treatments, only Biopolymers C and D were statistically different colors from the untreated stone after 30 days (Table 5).

The two-way ANOVA showed that there were no statistically significant differences in surface color variation over the 30 days ($F = 2.48$, $p = 0.065$). However, as demonstrated by the Student t tests, if time is considered independently, it becomes a factor involved in the total color change ($F = 19.08$, $p < 0.001$). However, only the treatment variable is not a factor promoting color variation ($F = 0.15$, $p = 0.979$). The interaction between both factors is decisive in the results; the values obtained ($p = 0.065$) are within the permissible limits to be considered statistically different, which may indicate that a longer time of exposure to natural conditions could make the color change more evident.

However, all ΔE values, including the control, were below the incompatibility value of $\Delta E > 5$ units, indicating that the color of the surface was not significantly affected by the application of biopolymers (Prieto et al. 2010; López-Arce et al. 2013; Camacho-Chab et al. 2021).

Chemical characterization of biopolymer A

Biopolymer A was chosen for chemical analysis based on two criteria: (a) improvement of stone surface stability and (b) yield after fermentation. Partial analysis of the hydrolysis products of the biopolymer showed that it contains both neutral (rhamnose, arabinose, and xylose/galactose/mannose) and acidic sugars (galacturonic and glucuronic acids). It was not possible to determine the identity of the neutral monosaccharide (xylose/galactose/mannose), since these three monosaccharides could be co-eluting and therefore appear as an overlap in the chromatographic profile (Fig. 5). The major monosaccharide was galacturonic acid (64.1%), followed by arabinose (13.4%) and glucuronic acid (11.7%), with minor amounts of other sugars, including one unidentified (Table 6).

Discussion

Limestone is particularly prone to disaggregation and surface exfoliation (Bonazza et al. 2017), and it is therefore important to develop an effective and environmentally acceptable consolidation treatment for historic limestone monuments. Consolidants have been used to lessen stone deterioration processes, such as scaling, flaking, spalling, and granular disintegration, that contribute to mechanically weakened stone. Restorers are generally reluctant to use synthetic consolidants because they are not readily reversible and may also pose health risks to humans (Wheeler 2005). This is one of the main reasons for the increasing interest in using bio-based materials as potential novel consolidants (Ortega-Morales and Gaylarde 2021).

In this study, a preliminary assessment was conducted to assess the influence of bacterial biopolymers on the physical properties of experimental limestone blocks, determining the surface consolidation effect based on the peeling test (disaggregation) and hardness measurements. Selected biopolymers (A, B, C, and D), synthesized by bacterial isolates TM1B-488, TM1B -489, TM1B-349, and TM1B-464, positively influenced surface disaggregation and hardness at different levels. When compared with an untreated limestone control, biopolymer A reduced the surface disaggregation of material by 26%, while biopolymer B induced a lower protection effect (10%), and the remaining biopolymers (C and D) only had a negligible effect. Cactus (*Opuntia* sp.) biopolymer, used as a known positive control, reduced disaggregation at similar, but higher, levels (37%). The use of biopolymers (mucilage) from the *Opuntia* genus to increase

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the aggregation of mortars particles has been reported previously (Alisi et al. 2021).

A recent study by Elert et al. (2021), in which they used biomineralization—the production of calcium carbonate by bacteria—for stone conservation, may be compared with our results. They showed that stimulating the activity of indigenous carbonatogenic bacteria reduced stone loss from a severely degraded Mayan tuff stone at Copan by 70%, measured three months after in situ treatment. They assessed stone loss using the same tape peeling method as described in the current article and showed that the reduction in stone disaggregation resulted from newly formed biocalcite as well as attached polymeric production by the activated native microbiota. Thus, this bioconsolidation effect appeared to be a synergistic action of both processes.

The increase in effectiveness of the Elert et al. (2021) treatment was apparently due to the presence of active carbonatogenic bacteria, with the attendant potential problems associated with live bacteria. However, it is also important to note that they used a highly degraded stone in their studies. Our samples were of sound stone, and yet biopolymer treatment not only reduced stone loss, as indicated by the peeling test, but also increased surface hardness by amounts ranging from 34% (biopolymer A), through 32% (biopolymer B), to 19% (biopolymer D) and 10% (biopolymer C).

Overall, our results are promising, considering that the biopolymer treatments were simply applied by brushing onto the surface of the limestone samples, a technique used by today's conservators. Consolidants are usually applied to the stone surface by brush or spray (Doehne and Price 2010). It is interesting to note that the Mayans were aware of the relevance of protecting their monuments and employed complex mixtures based on burnt limestone and biobased materials (eggs and plant extracts), for consolidation purposes (Litman 1959). The current trend to using biobased materials as admixtures thus dates back to ancient times and has contributed to improving the properties of mortars and plasters, increasing cohesion to surfaces.

It is important that any substance applied to the surface of a historic property of cultural importance does not alter its appearance. This is usually determined by a change in color. Our treatments did not cause a significant color change to the limestone samples; total color change was below the threshold value of $\Delta E < 5$, indicating a non-significant color alteration on the limestone surfaces over time. This finding is interesting as it shows that bacterial biopolymer-based coatings are compatible with limestone from the user's point of view, an important aspect to consider in the color stability of polymer-treated surfaces. Kaplan et al. (2019) showed that synthetic polymeric coatings of Paraloid B72 modified the surface color of rocks, with values of ΔE above the permissible threshold. Similarly, while the coatings PURASORB PL 65 (PLA) and polyhydroxybutyrate (PHB), a biopolymer, were found initially to alter color below the acceptable chromatic alteration threshold, this ability was modified after 2250 h. Our biopolymer-coated limestone did not induce a significant chromatic alteration, at least after 30 days, but the results of Kaplan et al. (2019) suggest that this time should be extended. To our knowledge, no previous research has reported such negligible color changes after coating stones with bacterial biopolymers.

Molecular biology-based identification (complete 16S rRNA gene sequences) showed that isolate TM1B-349 and TM1B-464 were highly related with *Psychrobacter* sp. strain Marseille P-5312 and *Agrococcus terreus* strain BT116, respectively. Whereas isolates TM1B-488 and TM1B-489 had over 99% identity with *Xanthomonas citri* pv. mangiferaeindicae strain XC01. Bacteria of the genus *Agrococcus* have been previously reported for stone environments (Elert et al. 2021). In contrast, *Xanthomonas citri*, a known phytopathogen and *Psychrobacter* from cold environments, have not been, to our knowledge, reported for lithic surfaces. In agreement with previous studies, representatives of the three genera possess the ability to synthesize biopolymers (Moreira et al. 2001; Patel and Parikh 2011; Jroundi et al. 2020; Jabeen et al. 2022).

Microbial bioprospecting has been an active field to detect, isolate and conserve microorganisms with applications in food, industrial, medical, and environmental fields (Verma and Shah 2022). There is limited knowledge of the actual or potential use of environmental microorganisms in cultural heritage conservation (Mitchell and McNamara 2010). The selection of microorganisms with the desired properties (e.g., biomineralization) is one of the most important steps. During our study, only 4 out of 13 bacterial isolates displayed an EPS-producing phenotype, a carbohydrate content of EPS, the ability to grow in a liquid medium and to yield significant amounts of EPS, in the order of g L^{-1} (Freitas et al. 2017). Our partial chemical characterization of the best-performing biopolymer (A) suggests a probable glycoprotein nature, whose constitutive acidic monosaccharides likely contributed to higher adherence to the limestone surfaces, contributing to surface stabilization, hardening, and decreasing decohesion. The percentage of the chemical fraction not detected by the colorimetric tests may be associated with the cell debris or be present as other molecules, such as lipids, extracellular nucleic acids, or low molecular weight molecules.

Biopolymers derived from epilithic bacteria or associated with epilithic microbial biofilms may enhance or inhibit stone decay (Decho 2010). In our study, the selected bacterial biopolymers contributed to stone conservation. The nature of the polymers is important in determining their action on stone surfaces; molecular weight and secondary and tertiary structure play a part (Flemming and Wingender 2010); the acidic nature of some

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polysaccharides may contribute to enhanced adherence to limestone surfaces, consolidating stone by participating in calcium complexation. Our biopolymers have not yet been completely analyzed and it cannot be ruled out that they are a mixture of polymers.

If future studies prove the applicability of bacterial polymers as limestone consolidants, this will add to their current list of applications. Their thickening, stabilizing, and aggregation properties (Ercole et al. 2012) have found use in several preparations, including cosmetics and soil additives (Thankachen et al. 2021). There is recent interest in their applications in the building industry. Nevertheless, biobased consolidants face challenges for future and widespread application. They offer an easy, cheap, and technically feasible product for non-specialists, but they possess a major drawback as consolidants, represented by their biodegradability; they can act as carbon sources that could induce the activation of deleterious acid-producing bacteria.

Overall, as a concluding remark, this study showed that biopolymers derived from epilithic bacteria can contribute to the consolidation. This consolidation was assessed with simple techniques. This reinforces the value of using non-destructive techniques to assess the physical properties of stone under treatment and the need to spread the knowledge of transdisciplinary approaches to advance the science and technology of cultural heritage conservation.

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Author contributions JCCC: Conceptualization, formal analysis, investigation, writing—review and editing. JEPS: Methodology, formal analysis, investigation, writing and editing. PACC: Methodology, formal analysis, investigation. CG: Methodology: Formal analysis, writing—review and editing. MLAO: Investigation, writing and editing. JMOA: Methodology, investigation, review and editing. MJCB: Methodology, formal analysis, investigation. PQO: Methodology, formal analysis, investigation. BOOM: Conceptualization, founding acquisition, project administration, supervision, writing-review and editing.

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Data availability All data generated or analyzed during this study are included in this manuscript.

Declarations

Conflict of interest The authors declare that they have no know competing interest or personal relationship that could have influenced the work reported in this paper.

Ethical approval This study does not contain any experiments with human participants or animals.

Consent to participate All co-authors agreed to be integrated into the research.

Consent to publish The publication has been approved by all co-authors.

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