

PRODUCTION OF GREEN BIOCIDES FOR CULTURAL HERITAGE. NOVEL BIOTECHNOLOGICAL SOLUTIONS

Mara SILVA^{1,2}, Tânia ROSADO², Dora TEIXEIRA^{1,2},
António CANDEIAS^{1,2} Ana Teresa CALDEIRA^{1,2*}

¹Chemistry Department, School of Sciences and Technology, Évora University,
Rua Romão Ramalho 59, 7000-671, Évora, Portugal

²HERCULES Laboratory, Évora University, Largo Marquês de Marialva 8, 7000-809 Évora, Portugal

Abstract

The growth control of microflora in cultural and built heritage is usually performed by treatments using chemicals that have high toxicity. Thus it is crucial to develop strategies to discover new bioactive molecules and establish effective approaches against the effectively biodeteriogenic agents, which are responsible for degradation of cultural and built heritage, without detriment to the environment. Bacillus sp. produce a high number of secondary metabolites, some with antibiotic properties which can be used against biodeteriogenic filamentous fungi, source of serious damage in historic materials. These antagonistic properties are due to the production of bioactive lipopeptides which exhibit antifungal activity in the stationary phase of bacteria growth, being associated to secondary metabolism. A combined methodology using antifungal tests, chromatographic techniques, FTIR-ATR, microscopic approaches and simulations assays allowed the detection of antifungal potential and a rapid identification of these ground-breaking bioactive compounds without the need of previous total isolation. This novel green biocides show a great potential for future application in cultural and built heritage rehabilitation being an effective alternative to the chemical compounds usually applied.

Keywords: Cultural Heritage; Bioactive compounds; Lipopeptides; Bacillus sp.; Biocides

Introduction

Biological colonization by microbial agents are an undesirable process that can affect cultural and built heritage and economically important materials. Although several biotic and abiotic conditions such as humidity, temperature, light and chemical factors like the nature of the substratum can accelerate this process, microorganisms are perhaps its main promoters [1]. Many microorganisms such as bacteria, fungi, algae and lichens produce serious damage in historic materials, which are decomposed by the action of specific enzymes and organic acids produced by microbial communities [2]. Lichens however play a minor role in colonization, algae and bryophytes although, often abundant in the plasters and mortars, are considered less important in biodegradation but they support the colonization and development of allied heterotrophic population of bacteria and fungi which are the main biodeteriogens responsible for esthetical and structural damage [3]. Fungi are particularly dangerous because their hyphae may have high level of proliferation and their spores, in a dormant state, are commonly present

* Corresponding author: atc@uevora.pt

and available for germination. On the other end, fungal-derived carboxylic acids (e.g., oxalic, citric, succinic, formic, malic, acetic, fumaric, glyoxylic, gluconic, and tartaric acids) can play a significant role in chemical attack [4, 5].

Development of fungi in heritage artefacts can induce discoloration and deterioration of its surface, leading to the appearance of stains that alter the colour, on the other hand hyphae penetration may lead to detachment of fragments [6, 7]. The importance of carrying out proper remediation action for microbiologically contaminated historic materials is of vital importance.

Biocide treatments are considered to be one of the practical approaches for bioremediation and conservation of artworks [2]. Nevertheless, protective solutions based on the use of toxic chemical compounds that may be accumulated in animal tissues [8] face increasing restrictions. Innovative research is needed to replace the biocides currently used in conservation and restoration of art works by green solutions that are eco-friendly and do not present negative effects on the environment or human beings [9].

Bacillus species are worth for this treatment because they produce a great diversity of secondary metabolites with biological activity [10], namely they are well known to possess antagonistic activities against many fungal pathogens. Some strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been referred to produce antifungal peptides [11-13]. Usually, three different classes of bioactive peptides can be distinguished: antifungal peptides, such as bacilysin and rhozotocin; antifungal lipopeptides, such as surfactins, iturins and fengycins; and antimicrobial polypeptides, such as subtilin [14].

The structure of those lipopeptides present a cyclic peptidic portion with seven amino acids (iturin and surfactin) or ten aminoacids (fengycin and plipastatin), linked to the fatty acid chain. The length of the fatty acid chain can vary, originating different forms of these compounds for each lipopeptide [15].

The synthesis of lipopeptide compounds are common in nature because the mechanism behind its production by some bacteria is directly related to defence to stress situations, like sporulation, these compounds can bring benefits to the individual. In nature, these lipopeptides increase the surface area from non-soluble hydrophobic growth substrates and the solubility of hydrophobic substances improving their biologic availability, and participate in the adherence and detachment of microorganisms from surfaces [16].

Materials and Methods

Microorganisms and inoculum preparation

The strains of *Bacillus* sp. CCMI 1051, CCMI 1052 and CCMI 1053 were isolated from healthy *Quercus suber* in the south of Portugal and identified according with morphological, physiological and biochemical characteristics and by 16S rDNA sequence analysis (accession number AY785773, AY785775 and AY785774, respectively) [17]. Cells were maintained on Nutrient Agar (HIMEDIA) slants and stored at 4°C. The fungal cultures were maintained on malt extract agar (HIMEDIA) slants and used as test microorganisms. The strains *Cladosporium* sp., *Penicillium* sp., *Fusarium oxysporum* and *Aspergillus niger* were isolated from biodegraded mural paintings and belonging to the laboratory collection (HERCULES-Biotec laboratory, Évora University).

Growth conditions and bioactive compounds production

The *Bacillus* sp. CCMI 1051, CCMI 1052 and CCMI 1053 cells were inoculated in 100 mL of Nutrient Broth (HIMEDIA) medium. To monitor the microorganism growth, liquid cultures were incubated for 72 hours at 30°C in an orbital shaker at 150 rpm (IKA KS 4000 I control) and the absorbance monitored at 600 nm.

The kinetics of the bacterial populations growth was modelled by the Gompertz model which is written as: $\log N/N_0 = A \cdot \exp(-\exp(-b-cx))$, where the N is the decimal logarithm of microbial counts (\log (CFU/mL)) at time t ; N_0 is the asymptotic log count as time decreases

indefinitely ($\log(\text{CFU/mL})$); A is the number of log cycles of growth ($\log(\text{CFU/mL})$); b is the relative growth rate at time (h^{-1}) and c is the time required to reach the maximum growth rate (h).

The specific growth rate, μ were determined from experimental data in exponential phase, using the relation $\mu (\text{h}^{-1}) = (c \cdot A)/e$, where μ is the specific growth rate of microorganism ($\log(\text{CFU/mL})/\text{h}$) and e is the *neper* number. The time of generation was calculated by $g (h) = \text{Ln}(2)/\mu$ [18]. The equation was fitted to triplicate sets of growth data using non-linear regression modules of the software SigmaPlot (Version 12.0.0, Germany).

For the production of bioactive compounds, the *Bacillus* sp. cells were cultivated in 2 L of NB medium in the same conditions as described previously. After 48 hours (stationary-phase) of culture growth, the bacterial cells were removed from the culture broth by centrifugation ($1,000 \times g$ for 10 min at 4°C). The two fractions were maintained at -20°C for further analysis.

Interaction between novel biocides and heritage biodeteriogenic fungi

In order to evaluate the antifungal activity of the biocides produced, a fungal spore suspension of each biodeteriogenic fungi in test was prepared suspending loopfuls of hyphae and spores in 5 ml of 0.9% NaCl solution. The spore suspension was obtained through a serial dilution and adjusted to 10^5 CFU/mL. A mixture composed by 5 mL of malt extract, 0.5 mL of 10^5 CFU/mL of spore suspension and 5 mL of culture broth was incubated for 24 h at 28°C . One millilitre of each interaction mixture was plated, by incorporation in 20 mL of Cooke Rose Bengal agar (HIMEDIA), and the Petri dishes were incubated at 28°C for 24–48 h. The relative inhibition against a control test (%) of the same fungus in the absence of the *Bacillus* sp. CCM1 1051 supernatant was determined by counting the number of colonies [17, 19].

Biocides extraction

Lipopeptides produced in liquid cultures were isolated from culture broth by adding 3M HCl to a final pH of 2.0. The acid precipitates were collected and extracted with methanol. The methanol soluble compounds were vacuum-dried and recovered for further analysis [20].

Antifungal activity of bioactive compounds

Antifungal paper disks diffusion assay

Fungal spore suspension of *Cladosporium* sp., *Penicillium* sp., *Fusarium oxysporum* and *Aspergillus niger* were prepared by adding loopful of hyphae and spores from a Malt Extract Agar (MEA) slant incubated at 25°C for 7 days, in 5 mL of NaCl 0.85% solution. The suspension was filtered by sterilized cotton or triple gauze. A 10^6 CFU/mL spore suspension was obtained through dilutions and fungal suspensions were incorporated in MEA at 45°C in Petri dishes. Filter paper discs (Macherey-Nagel 827 ATD) impregnated with 10 μL of *Bacillus* culture broth, after cells removal were placed on the agar and the Petri dishes were incubated at 25°C for 24–48 h. Antifungal activity was indicated by the formation of inhibition halos around the discs [21].

Bioautographic detection

The lipopeptides extracted were separated by thin layer reverse phase chromatography. A 25 μL of extracts in test (3 mg/mL) were applied on silica gel pre-coated TLC plates (0.2 mm, 20 x 20 cm, IF254, Merck, Darmstadt, Germany), eluted with chloroform:methanol:water (65:35:5) and dried in order to remove the solvent. UV-active compounds were detected at 254 and 360 nm and marked on the plates.

To evaluate the antifungal activity of the active compounds separate in TLC, a *Cladosporium* sp. cell suspension (10^6 cells mL^{-1}) were sprayed over the plates with the detected compounds and incubated at 25°C for 72 h, protected from light. Antifungal activity was indicated on the bioautograms by clear spots.

FTIR-ATR was used to monitor the chemical lipopeptidic nature of the biologically active compounds on the bioautographic assays.

Infrared spectroscopy was performed on an Alpha-R spectrometer from Bruker Optics, with an Attenuated Total Reflection (ATR) module. Bruker® OPUS 6.5 software was used for processing the spectra. The IR spectra were plotted in the region between 4000 and 350 cm^{-1} , with 128 scans and spectral resolution of 4 cm^{-1} .

in vitro assay using mortars slabs

To evaluate real life efficiency and influence of the new compounds on the activity of biodeteriogenic microorganisms, a combinatory strategy using the extracted bioactive molecules under *in situ* controlled conditions was performed.

Mortars slabs of 3 x 6 cm were manufactured in the laboratory using Vitruvius formulation [22]. Mortars were composed by two mixed binders, aerial lime and siliceous sand (1:3) and cured for two weeks.

After sterilization, two different assays were performed in parallel as shown in the methodological scheme (Fig.1).

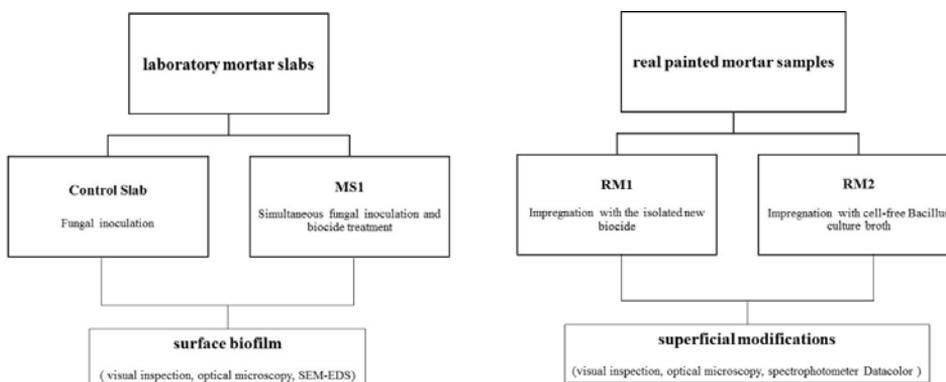


Fig. 1. Methodological scheme of simulation *in vitro* assay using mortars slabs and real painted mortars under the presence of the new biocides assay.

Slab mortars were inoculated with a mixture composed by 1 mL of *Cladosporium* sp. 10^5 UFC/mL spore suspension, 1mL of malt extract and 500 μL of the new biocide produced by *Bacillus* sp. CCM1 1053.

The slabs were incubated at 25°C, monitored periodically and documented using a digital camera. A control assay was also performed in the absence of the new biocides.

After 2 months of incubation all the slabs were air-dried, coated with gold and examined by scanning electron microscopy (SEM) using a Hitachi S-3700N (Tokyo, Japan) variable pressure scanning electron microscope coupled to a Bruker XFlash 5010 energy dispersive X-ray (EDS) spectrometer (Berlin, Germany) to allow visualization of the surface “biofilm” and elemental composition (point analysis and two-dimensional mapping). The accelerating voltage was 18-20 kV.

Real mortars under the presence of the new biocides

Real samples of a mortar containing mural painting was monitored in the presence of the new biocides, in order to evaluate the possible structural or superficial modifications after the applications of these novel compounds.

Two series of assays were performed using real mortars obtained from detached fragments (about 2x2cm) of a mural painting (Santo Aleixo Church, Montemor-o-Novo, Portugal) as described in Figure 1. The first series was prepared impregnating 200 μL of the isolated new biocide (3mg/mL) - RM1 series. The second series was prepared impregnating 500

μL of the cell-free culture broth from *Bacillus* sp. CCMI 1051 cultures, in the surface of the painted mortars - RM2.

During five months the possible surface alteration of the painted mortars was photographically documented. At the end of the assay, the possible modifications were recorded with Leica M205C stereozoom microscope, with a Leica DFC290HD camera, for image acquisition, and colour evaluation was performed with a spectrophotometer Datacolor CheckPlus II (DataColor, NJ). The results obtained in the CIE $L^*a^*b^*$ chromatic space defined by the CIE in 1976, are the average of five measurements taken on the paint layer surface. The chromatic coordinates measured were L^* that represents lightness (0–100); a^* that stands for the red/green axes, and b^* that stands for the yellow/blue hue axes (0–100). The diffuse reflection spectral curve of the pigments in the visible range (380–740 nm) was also measured.

Results and discussions

Bacterial growth characterization and bioactive molecules production

The species *B. amiloliquefaciens* selected for this work has been previously reported to produce compounds with antifungal proprieties against filamentous fungi that attack forest products. *B. amyloliquefaciens* CCMI 1051 cultures displayed antifungal activity due to the production of extracellular lipopeptides [12, 17, 21]. This paper presents the methodological basis and the proposal of a new alternative to the production of green biocides directed to heritage biodeteriogenic fungi. The three selected strains of *Bacillus* were cultured in liquid medium during 48 h.

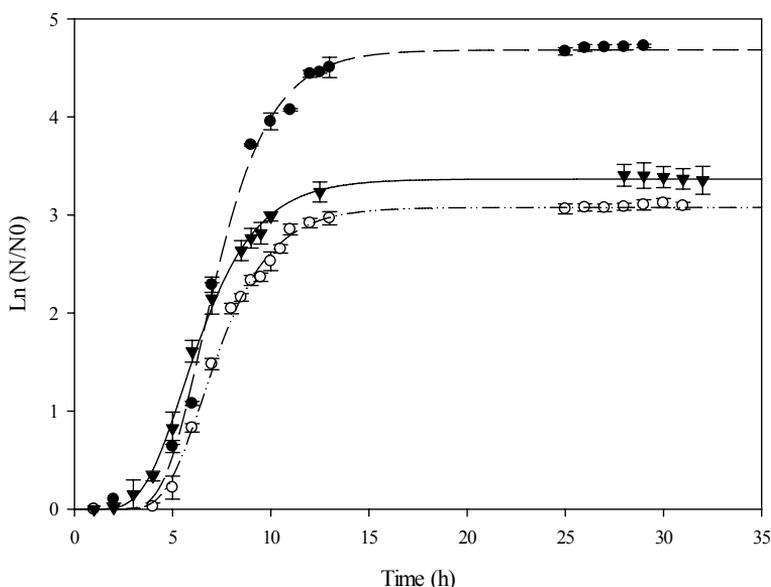


Fig. 2. Time course profiles of *Bacillus* sp. in test growth. All data was determined in triplicate. A - *Bacillus* sp. CCMI 1051 (○); *Bacillus* sp. CCMI 1052 (●); *Bacillus* sp. CCMI 1053 (▼).

Fig. 3 shows the time course profile of the three strains *Bacillus* sp. tested. All cultures present similar kinetic features, namely a lag phase during about 3 hours, a period of exponential growth of approximately 8-10 hours and a stationary phase, after 11 h of culture (Fig. 2). The specific growth rate and the generation time was determined from the exponential growth period and the *Bacillus* sp. CCMI 1051 display a major specific growth rate (μ) and a minor generation time (g) (Table 1).



Fig. 3. Antifungal activity of biocides against *Cladosporium* sp. using paper disk diffusion assay. A- Control. B- In the presence of 10 μ L of *Bacillus* sp. CCM 1053 cell free culture Broth.

Table 1. Specific growth rate and generation time values for the *Bacillus* sp. strains.

<i>Bacillus</i> sp.	Specific Growth Rate μ (h^{-1})	Generation Time g (h)
CCMI 1051	0.893 ± 0.102	0.776 ± 0.188
CCMI 1052	0.594 ± 0.060	1.168 ± 0.021
CCMI 1053	0.603 ± 0.056	1.149 ± 0.095

The bioactive compounds from the secondary metabolism of each bacteria were recovered on stationary phase when nutrients starvation limit the growth. Starvation can be met by a variety of alternative survival strategies corresponding to the production of secondary metabolites that can act as surfactants, increasing the surface area from non-soluble hydrophobic growth substrates and the solubility of hydrophobic substances [23].

Antifungal activity of bioactive compounds

After 24h of culture, the supernatants of liquid cultures were tested against the biodeteriogenic fungal strains isolated from mural paintings: *Cladosporium* sp., *Penicillium* sp., *Fusarium oxysporum* and *Aspergillus niger*.

Antifungal assays show a higher inhibition level for the *Bacillus* sp. CCM 1053 cultures against *Cladosporium* sp., confirmed by the formation of an inhibition halo of 37.44 ± 1.83 mm (Fig. 3 and Table 2).

Table 2. Inhibition halos of *Bacillus* sp. against the biodeteriogenic fungi

<i>Bacillus</i> sp.	Inhibition halos (mm)			
	<i>Aspergillus niger</i>	<i>Fusarium oxysporum</i>	<i>Penicillium</i> sp.	<i>Cladosporium</i> sp.
CCMI 1051	21.22 ± 2.69	20.00 ± 0.33	21.44 ± 0.22	22.11 ± 0.19
CCMI 1052	12.78 ± 0.92	18.56 ± 0.69	12.72 ± 0.08	17.33 ± 0.03
CCMI 1053	21.44 ± 0.69	22.09 ± 0.51	20.03 ± 0.14	37.44 ± 1.83

The results of the inhibition assays, corresponding to the stationary-phase of bacteria growth allow to verify that *Bacillus* sp. CCM 1053 has a height inhibitory capacity against the biodeteriogenic fungi tested, however with different levels of antifungal capacity depending on the biodeteriogenic target fungal strain.

Results of the liquid interaction assays (Fig. 4) reveal that *Aspergillus niger* was the less inhibited fungi, nevertheless their inhibition release 70% when in interaction with supernatants of *Bacillus* sp. CCM 1052 cultures. Particularly high antagonistic activity nearly to 100% was detected for all bacillus cell-free supernatant of cultures, against *Fusarium oxysporum*, *Penicillium* sp. and *Cladosporium* sp. The four heritage biodeteriogenic fungi tested can be inhibited in the presence of the new biocides produced by bacterial cultures, showing the great potential of this biotechnological approach applied to heritage context.

In order to evaluate the antifungal activity directly on the compounds, without the need of total isolation, the biocides were extracted from *Bacillus* sp. CCMI 1052 cultures, separated by thin layer reverse phase chromatography and used in a bioautographic detection. FTIR-ATR was used to monitoring the chemical lipopeptidic nature of the biologically active compounds on the bioautographic assays against *Cladosporium* sp.

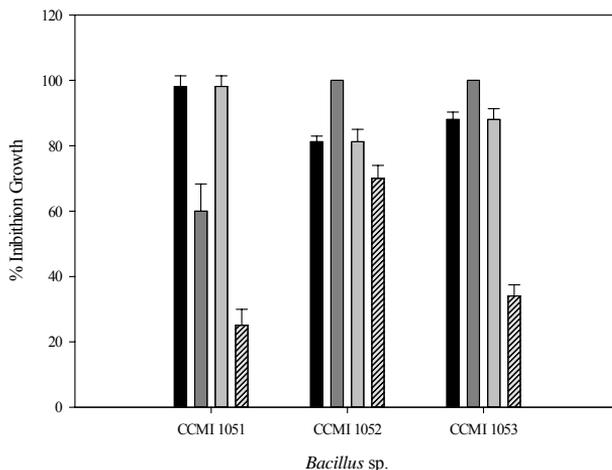


Fig. 4. % of inhibition growth resulted from interaction assays between biodeteriogenic fungi and *Bacillus* sp. cell-free culture broth. ■ *Fusarium oxysporum*; ■ *Cladosporium* sp.; ■ *Penicillium* sp.; ▨ *Aspergillus niger*.

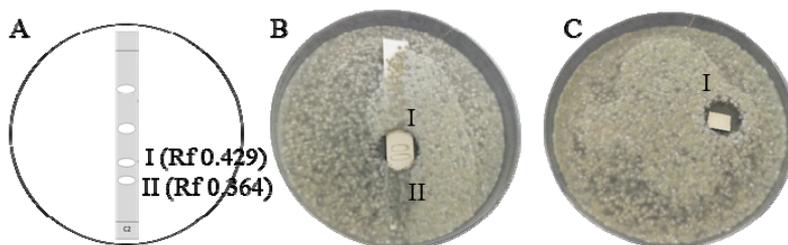


Fig. 5. Autobiographical results. Chromatographic scheme profile (a). The *Bacillus* sp. CCMI 1052 extracted cell-free supernatant shows the presence of two antifungal compounds against biodeteriogenic fungi *Cladosporium* sp. B – Scheme of TLC plate in petri dish with the two layers (I- Rf = 0.429 and II- Rf = 0.364); C- Bioautograms with TLC layers applied separately from *Bacillus* sp. CCMI 1052

TLC shows layers with different Retention Factor - Rf (0.364 and 0.429) detected on a UV chamber at 254 nm and 365 nm. Fig. 6 shows the chromatographic scheme profile (Fig. 5A) and the bioautogram correspondent, obtained from the chromatographic plate (Fig. 5B) and from the cropped spots (Fig. 5C). The clear spots with Rf values of 0.364 and 0.429 corresponding to antifungal compounds produced were analysed by FTIR-ATR directly on the silica plate (Fig.6).

The more evidenced peak centred at 1090 cm^{-1} corresponds to the silica background, which is present in the matrix of TLC plate. The compounds showed a broad absorbance peak around 3645 cm^{-1} , ranging from $3500\text{ to }3740\text{ cm}^{-1}$, a typical feature of compounds containing carbon and amino groups [24]. These peaks can be attributed to the stretching vibration of C–H and N–H bonds and intramolecular hydrogen bonding. The peak centred at 3744 cm^{-1} was attributed to the stretching of O–H bonds of the carboxyl groups, peak 2980 cm^{-1} , indicate the presence of C–CH₃ bonding or long alkyl chains typical of lipopeptide compounds [25]. The

peak in the region from 1520 cm^{-1} a 1555 cm^{-1} , reveal the presence of C=O bonds and its stretching vibrations. These results established that the antifungal compounds possess a lipopeptidic nature. LC-ESI-MS confirm the lipopeptide presence on the total extract (results not shown).

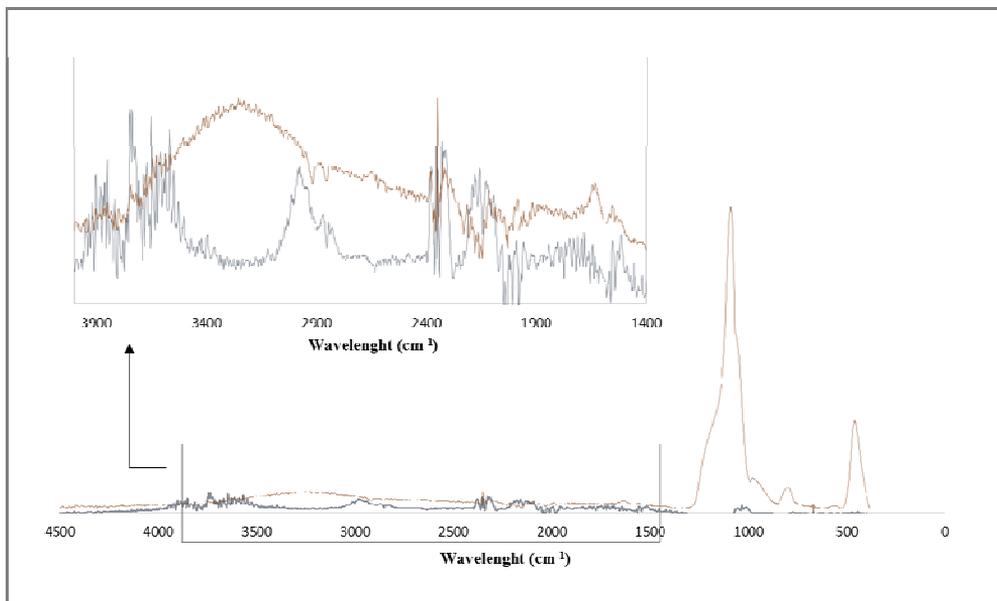


Fig. 6. FTIR-ATR analyses of the biologically active compounds produced by *Bacillus* sp. CCM1 1052 on the bioautographic plate. — *Bacillus* sp. CCM1 1052; — Silica

Simulation in vitro assays using mortars slabs

In order to develop and study the real life efficiency and influence of these new biocides in the growth of biodeteriogenic fungi, a combinatory strategy using manufactured mortars slabs inoculated with *Cladosporium* sp. and treated with extracted bioactive compounds, under *in situ* controlled conditions were envisaged.

Fig. 7 shows the macroscopic and microscopic features corresponding to the mortar slab assays in the presence of *Cladosporium* sp.

After 2 months of mortar slab treatment, the positive control show dark stains in the painted surface, indicating a high level of visual fungal contamination (Fig. 7A). The MS assay with antifungal treatment presented a very low level of visual fungal proliferation and no signals of cracking or detachments were detected on the surface of the slabs (Fig. 7F). In order to confirm the previous result, microfragments of the two series of mortars slabs were analysed by Scanning Electron Microscopy (SEM). This technique provides images of high magnification and resolution and allows to infer about the existent contamination by direct observation. The control slabs (Fig. 7B, C) exhibit a clear presence of microbial cells thriving in the mortar. It is possible to observe *Cladosporium* hyphae and reproductive structures penetrating in the microstructure of the mortars, promoting the proliferation of these microorganisms in depth. Also, the EDS elemental analysis show the presence of elements such as carbon, nitrogen and oxygen, confirming the presence of organic material in the slab (Fig. 7D, E). Regarding to biocide treatment in MS slabs, no fungal growth was noticed, with a total lack of spores and hyphae also confirmed by the EDS analyses that only show the presence of mortar elements (Fig. 7G, H).

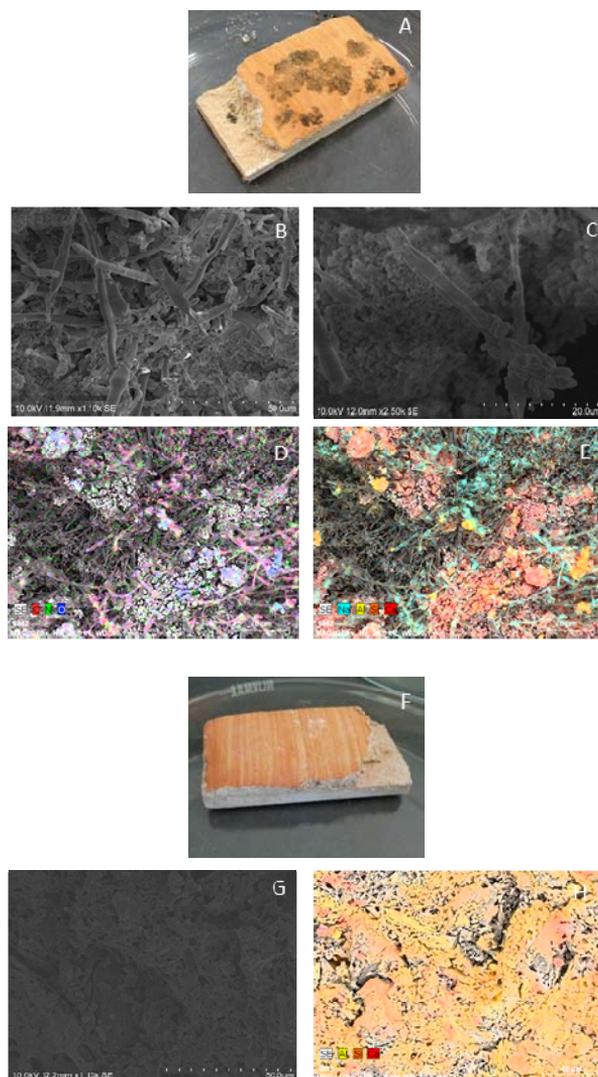


Fig. 7. Slabs mortars, SEM micrograph of the MS microfragments and EDX analyses. A,B,C,D,E -Control slabs inoculated with *Cladosporium* sp.; F,G,H- MS with bioactive compounds produced by *Bacillus* sp. CCMMI 1053.

Real mortars monitorization

Small and representative fragments of mural painting was monitored and analysed in the presence of the bioactive compounds produced to simulate the impact and possible alteration resulting from the new biocide treatment in the microstructure of the mortar and on the painting. Fig. 8 show the two pieces of real mortars that were impregnated with extracted compounds (RM1) and cell-free supernatant from *Bacillus* sp. CCMMI 1053 culture (RM2) which proved to be one of the most efficient strain for the bioactive compounds production.

After 5 months incubation under controlled conditions, the two series of mortars do not present any pigments discoloration signals, cracking and detachments in its structure (Fig. 8).

Fig. 9 provides an overview of the current palette of the control and two series of real mortars impregnated with the novel biocide under study, projected on the CIE L* a* b* colour space. The a* values represent the red and green chromatic coordinates and b* the yellow and blue. The results show a predominance of red in both series and the control mortar ($6 < a^* < 16$)

and show no significant differences between control and treated mortars ($p>0.5$). Therefore this new biocide does not cause any structural and aesthetics damage both in painting and mortar, becoming an appropriate treatment with great potential to be applied in cultural and built heritage as treatment or prevention of microbial grow.

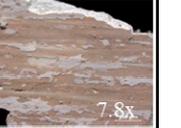
	RM1		RM2	
	Before impregnation	5 months later	Before impregnation	5 months later
Macroscopical features				
Microscopical features				

Fig. 8. Schematic representation of the lab experiments conducted on real mortars samples. RM1- Mortar impregnated with extracted compounds from *Bacillus* sp. CCMI 1053; RM2- Mortar impregnated with cell-free culture broth.

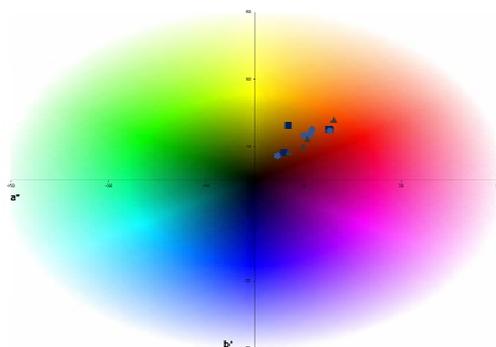


Fig. 9. Bi-dimensional projection of International Commission on Illumination a* b* chromatic coordinates of the 12 measurements that were performed in the real mortars of mural painting. ■ Control; ● RM1; ▲ RM2.

Few studies refer several negative aspects of chemical biocides application in artefacts and built heritage during the conservation and restoration process, namely the importance of restrain the amount of potentially dangerous biocides applied [26], the ineffectiveness of long-term treatment [27], the absence of selectivity of the target microorganisms [28, 29], discoloration and others structural damages in the economically important materials [30]. Hence, this new effective biocide specifically produced to biodeteriogenic agents of architectural, archaeological and artistic heritage obtained from natural sources have all the potential to become a ground-breaking mitigation solution to be applied in preventive and conservation treatment of artworks.

Conclusions

The *Bacillus* sp. CCMI1051 produce biosurfactant lipopeptides that suppress growth of heritage biodeteriogenic fungi. A combined methodology using antifungal tests, microscopic approaches and simulations assays allowed the detection of antifungal potential and a rapid identification of the bioactive compounds without the need of previous total isolation. These compounds can be used as additives for production of novel biocides for conservation and

restoration, constituting an efficient and environmental safe solution for biodegradation/biodeterioration of cultural heritage

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