GREEN BIOACTIVE COMPOUNDS: MITIGATION STRATEGIES FOR CULTURAL HERITAGE

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1. Introduction

The preservation of historic monuments and buildings constitutes a high societal priority in order for future generations to continue to witness their ancestors' achievements [1].

Biodeterioration phenomena represent a combination of physical and chemical alteration processes due to microorganisms in various materials, such as those constituting the objects that represent our cultural heritage [2]. Despite the involvement of several microorganisms in the deterioration process, the specific role of fungi is particularly central due to their high level of proliferation that can induce severe chemical attack in the materials [3-4]. Only the identification of the microbial communities associated with the different materials and the understanding of the role of such communities in the biodeterioration processes will enable the prevention and/or remediation of the problems related to bio-decay [5]. Several authors have reported biocide characteristics to eliminate biodeteriogenic organisms on stone monuments [6-8]. However, in recent years many of the most effective biocides have been banned due to their environmental and health hazards [7, 9].

Thus, the development of accurate remediation actions for microbiologically contaminated historic materials, based on environmentally safe solutions, is of vital importance.

Bacillus species are emerging as a promising alternative for built heritage treatment and rehabilitation due to their capacity to produce a great diversity of secondary metabolites with biological activity [10-11] such as surfactin, fengycin and iturin. The nontoxic mechanisms of action of these amphiphilic cyclic biosurfactants are therefore directly related, having unique features, high biodegradability, and non-harmful and environmentally friendly characteristics [12].

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Another alternative are medicinal plants, which have been widely used against pathogenic microorganisms in many parts of the world due to the effective antimicrobial activity of their extracts, such as essential oils. Plants of the Brazilian Cerrado, a tropical highland savanna in the midwestern region of Brazil have been broadly used in popular medicine. This region is characterized by an enormous range of extracts and essential oils with proven potential antifungal activity [13-15]. Several plants found in this biome belonging to different families, e.g. *Apocynaceae, Sapotaceae, Fabaceae*, are known for their antimicrobial, anti-inflammatory and antifungal activity [16-17]. One of them, *Pouteria ramiflora* (*Sapotaceae*) is known for its edible fruits and medicinal properties and is used in the treatment of obesity, helminthiasis, dysentery or inflammation. The literature reports different biological activities for this plant; however, few studies about its antifungal properties are described.

This work combines the potential of these two natural sources of green active compounds and intends to develop a natural green alternative to chemical biocides, thus contributing to a sustainable and environmentally safe conservation-restoration process for built cultural heritage.

2. Materials and methods

2.1. Bacillus bioactive compounds antifungal activity assessment

The *Bacillus* sp. CCLBH 1053 (Genbank accession number AY785774) were inoculated in 100 mL NB (Nutrient Broth, HIMEDIA) media for 72 hours at 30ºC in an orbital shaker at 150 rpm. After 48 hours (stationary-phase) of culture growth, the bacterial cells were removed from the culture by centrifugation and the secondary metabolites with antifungal potential (BEVOTECH 3, BEVOTECH 4, BEVOTECH 5) were maintained at -20ºC for further analysis [18, 11]. The biodeteriogenic strains isolated from biodegraded mural paintings, *Penicillium glandicola* CCLBH-MP101, *Alternaria* sp. CCLBH-MP401, *Fusarium oxysporum* CCLBH-I302 and *Mucor* sp. CCLBH-AA501 and belonging to the laboratory collection (HERCULES Biotech laboratory, Évora University) were used to perform antifungal tests to evaluate their potential to inhibit fungal proliferation. These assays were conducted according to previous study [11].

2.2. Pouteria ramiflora extracts antifungal activity assessment

2.2.1. Toxicity assay in Artemia salina

The *Pouteria ramiflora* extracts used in this work were provided by the research group of the Laboratory of Natural Products, Faculty of Health Sciences, University of Brasilia (UnB). The aqueous (AE) and ethanolic extracts (EE) used in antifungal assays were prepared by drying and powdering the *Pouteria ramiflora* leaves at room temperature. The ethanol crude extract was obtained by the maceration of plant material (40 g) at room temperature for seven days, with ethanol (2 L). After filtration, the solvents were removed under reduced pressure. The aqueous crude

extract was obtained by infusion. After the filtration procedure, water was removed by lyophilization [19].

The toxicity of the EE and AE was evaluated using the *Artemia salina* test kit (Artoxkit MTM, Microbiotest). The *Pouteria ramiflora* extracts were tested in a range of concentrations 50-3125 mg/L to establish lethal concentrations (LC_{50} – lethal concentration 50%) [20, 9].

2.2.2. Antifungal activity assay

The evaluation of the antifungal activity of *Pouteria ramiflora* extracts against biodeterogenic fungi isolated from artworks, *Cladosporium* sp. CCLBH-MP601, *Ulocladium* sp. CCLBH-EP701, *Fusarium tricinotum* CCLBH-MP303, *Penicillium* sp. CCLBH-EP102 and *Aspergillus niger* CCLBH-MP202, was carried out using antifungal disc diffusion assay [10, 9]. The results of this assay were represented according to a qualitative classification: +/− not determined (inhibition halo >12.7 mm), + positive test (inhibition halo = 12.7 mm), ++ (inhibition halo <12.7 mm). The microdilution method was conducted according to (Clinical Laboratory Standards Institute (CLSI) standard M27-A3) [21], using a biodeteriogenic yeast isolated from a mural painting, *Rhodotorula* sp. CCLBH-YMP502,

3. Results and discussion

The methodologies and products used for the conservation of artworks must be chosen bearing in mind the microbial population and the environmental impact, avoiding negative effects on/in the materials. Many strains are known to suppress fungal growth due to the production of antifungal antibiotics [22-27] especially cyclic lipopeptides that show a great potential for biotechnological, biopharmaceutical and agricultural applications.

The antifungal capacity of bioactive compounds produced by *Bacillus* strains was confirmed by means of antifungal tests to verify its efficiency as a green biocide capable of suppressing the proliferation of biodeteriogenic fungi on heritage.

Three formulations of bioactive compounds produced by *Bacillus* were tested against *Penicillium glandicola* CCLBH-MP101, *Alternaria* sp. CCLBH-MP401, *Fusarium oxysporum* CCLBH-I302 and *Mucor* sp. CCLBH-AA501. Figure 1 shows the antifungal activity results.

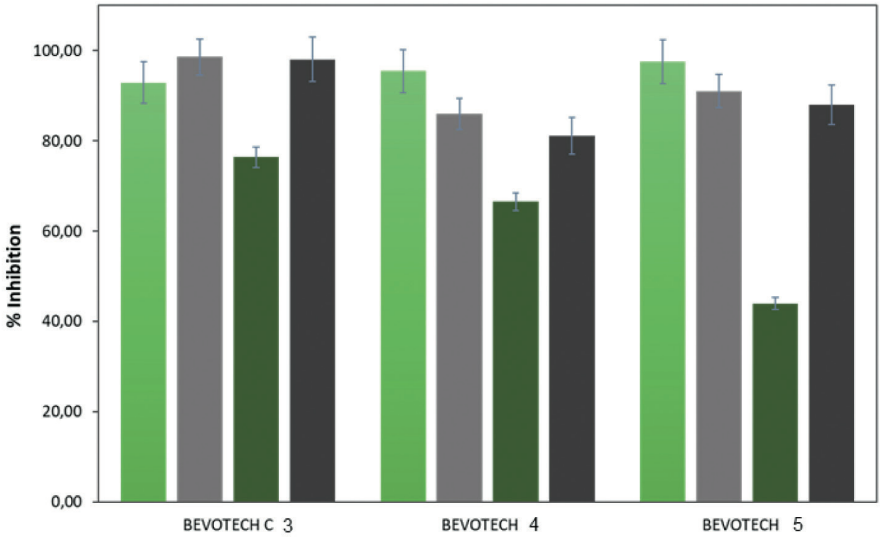

Figure 1. Antifungal activity of BEVOTECH 3, BEVOTECH 4, BEVOTECH 5 against biodeteriogenic fungi. Penicillium sp.; Alternaria sp.; Mucor sp.; Fusarium oxysporium.

The antifungal assay, using the *Bacillus* bioactive compounds BEVOTECH 3, BEVOTECH 4, BEVOTECH 5, showed a high inhibition level, independently of the fungal isolate tested, confirming previous studies that show the potential of BEVOTECH to be applied in the rehabilitation of cultural heritage and moreover, the absence of toxicity [11, 28, 9].

To find new, natural and effective solutions for treating biodegradation and broadening the spectrum of BEVOTECH's action, *Pouteria ramiflora* plant extracts from the Brazilian Cerrado which in previous studies showed a strong antifungal capacity in a medical context, were also tested [19, 29].

To evidence the non-toxicity of *Pouteria ramiflora,* both an ethanolic extract (EE) and aqueous extract (AE) were analysed using brine shrimp *Artemia salina* for the determination of LC_{50}. The results are presented in Figure 2.

The results show that with a concentration of 50 mg/mL the AE presents less toxicity than the EE and does not cause the death of any brine shrimp. However, for the highest concentration tested (3125 mg/L) both the EE and AE caused similar mortality (65% for AE and 63% for EE).

For both the EE and AE concentrations tested, none of the extracts caused 100 %-mortality. The AE fraction showed a LC_{50} of 1806 ± 1.03 mg / L and for the EE, the LC_{50} is close to 1560 mg / L. Both extracts proved to be considerably less toxic than chemical synthesis biocides used in the treatment and conservation of artworks, mainly AE [9], proving it to possess green characteristics and its potential to provide a valid alternative to the toxic chemical synthesis biocides usually applied.

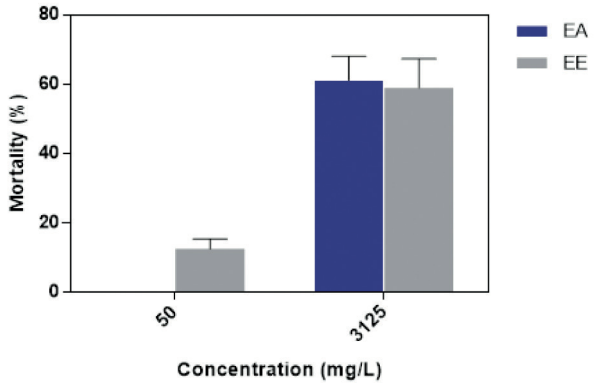

Figure 2. Toxicity of AE and EE at concentrations of 50 and 3125 mg/L in Artemia salina. (AE; EE).

In order to evidence the antifungal potential of both the EE and AE obtained from *Pouteria ramiflora,* in the context of cultural heritage, an antifungal assay was accomplished using five different filamentous fungi isolated from biodegraded artworks *Cladosporium* sp. CCLBH-MP601, *Ulocladium* sp. CCLBH-EP701, *Fusarium tricinotum* CCLBH-MP303, *Penicillium* sp. CCLBH-EP102 and *Aspergillus niger* CCLBH-MP202.

Table 1. Inhibition growth capacity of Ethanolic extract (EE) and Aqueous extract (AE) against biodeteriogenic fungi.

Biodeteriogenic fungi	EE			AE		
	12.5	**25**	**50**	**12.5**	**25**	**50**
Cladosporium sp. CCLBH-MP601	+	+	++	+/-	+/-	+
Ulocladium sp. CCLBH-EP701	+/-	+/-	+/-	+/-	+	+
Fusarium tricinotum CCLBH-MP303	++	++	++	++	++	++
Penicillium sp. CCLBH-EP102	+/-	++	++	+/-	++	++
Aspergillus niger CCLBH-MP202	+/-	+	++	+/-	+/-	+

+/− not determined (inhibition halo >12.7 mm; + positive test (inhibition halo = 12.7 mm), ++ (inhibition halo <12.7 mm)

The results show that *Fusarium tricinotum* CCLBH-MP303 was the most inhibited strain by the EE and AE (Table 1). Mostly, concentrations of 50 and 25 mg/mL for both extracts had an inhibitory action higher than 12.5 mg/mL. In the case of EE, the results showed a greater inhibitory capacity compared with AE. From a future perspective, a combination of EE and BEVOTECH may be considered to enhance the antifungal effect of both and thus create a totally green biocide with real effectiveness against fungal biodeteriogenic strains.

Additionally, the minimum inhibitory concentration (MIC) of the two crude extracts tested against biodeteriogenic yeast isolated from contaminated mortars *Rhodotorula* sp. CCLBH-YMP502 were also determined. The assay shows that both the EE and AE from *Pouteria ramiflora* present a MIC value of 6.25 mg/mL (Figure 3), a significantly low inhibitory concentration that evidences the potential of these extracts to be used to prevent the proliferation of this biodeteriogenic yeast.

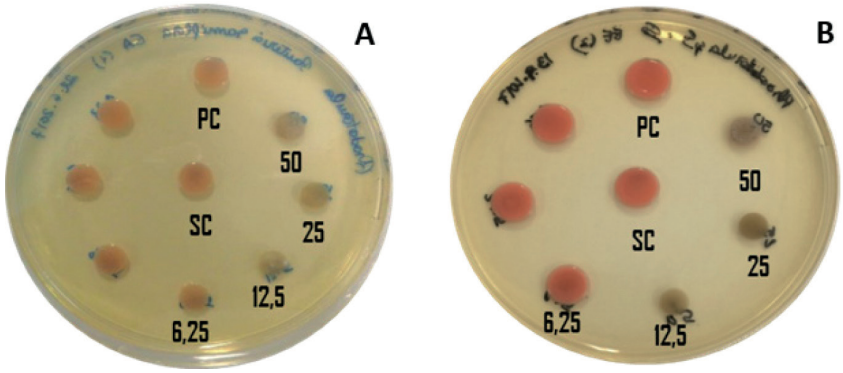

Figure 3. Minimum inhibitory concentration (MIC) of AE and EE against Rhodotorula sp. PC-Positive control; SC-Solvent control; A- Aqueous extract; B- Ethanolic extract

The results reveal the promising characteristic of the *Pouteria ramiflora* extracts to be used as an antifungal agent simultaneously against biodeteriogenic yeast and filamentous fungi, as its action is able to increase the effect of the Bevotech compounds.

Therefore, the antifungal activity of both natural products from *Pouteria ramiflora* and the bioactive compounds produced by *Bacillus* strains have shown they are effective against a set of filamentous fungi and yeast in a wide range of concentrations, thus making them prominent alternatives to be used separately or in a combined form as new additives for novel green biocides for cultural heritage. In conclusion, the tested products amplify the spectra of natural alternatives, paving the way to a new environmentally friendly biocide as an alternative to common chemical synthetized biocides, which can represent a serious risk for the environment, the artwork itself or even the conservator-restorer who handles it.

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References

[1] Steinbauer, M. J., Gohlke, A., Mahler, C., Schmiedinger, A., Beierkuhnlein, C., (2013) Quantification of wall surface heterogeneity and its influence on species diversity at medieval castles – implications for the environmentally friendly preservation of cultural heritage, *Journal of Cultural Heritage,* **14,** pp. 219-228.

[2] Pinzari, F., Pasquariello, G., De Mico, A. Biodeterioration of paper: A SEM study of fungal spoilage reproduced under controlled conditions. Macromolecular Symposia, 2006. Wiley Online Library, 57-66.

[3] Sterflinger, K., (2010) Fungi: Their role in deterioration of cultural heritage, *Fungal Biology Reviews,* **24,** pp. 47-55.

[4] Rosado, T., Gil, M., Mirão, J., Candeias, A., Caldeira, A. T., (2013) Oxalate biofilm formation in mural paintings due to microorganisms–A comprehensive study, *International Biodeterioration & Biodegradation,* **85,** pp. 1-7.

[5] Del Mar López-Miras, M., Martín-Sánchez, I., Yebra-Rodríguez, Á., Romero-Noguera, J., Bolívar-Galiano, F., Ettenauer, J., Sterflinger, K., Piñar, G., (2013) Contribution of the microbial communities detected on an oil painting on canvas to its biodeterioration, *PloS one,* **8,** pp. e80198.

[6] Milanesi, C., Baldi, F., Borin, S., Vignani, R., Ciampolini, F., Faleri, C., Cresti, M., (2006) Biodeterioration of a fresco by biofilm forming bacteria, *International Biodeterioration & Biodegradation,* **57,** pp. 168-173.

[7] Young, M. E., Alakomi, H. L., Fortune, I., Gorbushina, A. A., Krumbein, W. E., Maxwell, I., Mccullagh, C., Robertson, P., Saarela, M., Valero, J., Vendrell, M., (2008) Development of a biocidal treatment regime to inhibit biological growths on cultural heritage: BIODAM, *Environmental Geology,* **56,** pp. 631-641.

[8] Bastian, F., Alabouvette, C., Jurado, V., Saiz-Jimenez, C., (2009) Impact of biocide treatments on the bacterial communities of the Lascaux Cave, *Naturwissenschaften,* **96,** pp. 863-8.

[9] Silva, M., Salvador, C., Candeias, M. F., Teixeira, D., Candeias, A., Caldeira, A. T., (2016) Toxicological assessment of novel green biocides for cultural heritage., *International Journal of Conservation Science,* **7,** pp. 265-272.

[10] Caldeira, A. T., Feio, S. S., Arteiro, J. M. S., Roseiro, J. C., (2006) Antimicrobial activity of steady-state cultures of Bacillus sp CCMI 1051 against wood contaminant fungi, *Biochemical Engineering Journal,* **30,** pp. 231-236.

[11] Silva, M., Rosado, T., Teixeira, D., Candeias, A., Caldeira, A. T., (2015) Production of Green Biocides for Cultural Heritage. Novel Biotechnological Solutions., *International Journal of Conservation Science 6, SI 2015: 519-530***,** pp.

[12] Raaijmakers, J. M., De Bruijn, I., Nybroe, O., Ongena, M., (2010) Natural functions of lipopeptides from Bacillus and Pseudomonas: more than surfactants and antibiotics, *FEMS Microbiol Rev,* **34,** pp. 1037-62.

[13] Silva, E., Melo, F., De Paula, J. E., Espindola, L. S., (2009) Evaluation of the antifungal potential of Brazilian Cerrado medicinal plants, *Mycoses,* **52,** pp. 511-517.

[14] Souza, P. M., Elias, S. T., Simeoni, L. A., De Paula, J. E., Gomes, S. M., Guerra, E. N. S., Fonseca, Y. M., Silva, E. C., Silveira, D. M., Magalhaes, P. O., (2012) Plants from Brazilian Cerrado with potent tyrosinase inhibitory activity, *PLoS One,* **7,** pp. e48589.

[15] Zambuzzi-Carvalho, P. F., Tomazett, P. K., Santos, S. C., Ferri, P. H., Borges, C. L., Martins, W. S., De Almeida Soares, C. M., Pereira, M., (2013) Transcriptional

profile of Paracoccidioides induced by oenothein B, a potential antifungal agent from the Brazilian Cerrado plant Eugenia uniflora, *BMC microbiology,* **13,** pp. 227.
[16] Silva, C. A., Simeoni, L. A., Silveira, D., (2009) Genus Pouteria: Chemistry and biological activity, *Revista Brasileira de Farmacognosia,* **19,** pp. 501-509.
[17] Albernaz, L. C., De Paula, J. E., Romero, G. a. S., Silva, M. D. R. R., Grellier, P., Mambu, L., Espindola, L. S., (2010) Investigation of plant extracts in traditional medicine of the Brazilian Cerrado against protozoans and yeasts, *Journal of ethnopharmacology,* **131,** pp. 116-121.
[18] Caldeira, A. T., Feio, S. S., Arteiro, J. M., Coelho, A. V., Roseiro, J. C., (2008) Environmental dynamics of Bacillus amyloliquefaciens CCMI 1051 antifungal activity under different nitrogen patterns, *J Appl Microbiol,* **104,** pp. 808-16.
[19] Correia, A. F., Silveira, D., Fonseca-Bazzo, Y. M., Magalhaes, P. O., Fagg, C. W., Da Silva, E. C., Gomes, S. M., Gandolfi, L., Pratesi, R., De Medeiros Nobrega, Y. K., (2016) Activity of crude extracts from Brazilian cerrado plants against clinically relevant Candida species, *BMC Complement Altern Med,* **16,** pp. 203.
[20] Salvador, C., Martins, M., Candeias, M., Karmali, A., Arteiro, J. M., Caldeira, A. T., (2012) Characterization and biological activities of protein-bound polysaccharides produced by cultures of Pleurotus ostreatus, *Journal of Agricultural Science and Technology A,* **2,** pp. 1296-1306.
[21] Clinical and Laboratory Standards Institute. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; Approved Standard-Third Edition. CLSI document M27-A3. Wayne: Clinical and Laboratory Standards Institute Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; Approved Standard-Third Edition. CLSI document M27-A3, (2008). Wayne: Clinical and Laboratory Standards Institute.
[22] Leclère, V., Béchet, M., Adam, A., Guez, J.-S., Wathelet, B., Ongena, M., Thonart, P., Gancel, F., Chollet-Imbert, M., Jacques, P., (2005) Mycosubtilin Overproduction by Bacillus subtilis BBG100 Enhances the Organism's Antagonistic and Biocontrol Activities, *Applied and Environmental Microbiology,* **71,** pp. 4577-4584.
[23] Mukherjee, A. K., Das, K., (2005) Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by Bacillus subtilis strains in a particular habitat, *FEMS Microbiol Ecol,* **54,** pp. 479-89.
[24] Hsieh, F. C., Lin, T. C., Meng, M., Kao, S. S., (2008) Comparing methods for identifying Bacillus strains capable of producing the antifungal lipopeptide iturin A, *Curr Microbiol,* **56,** pp. 1-5.
[25] Kim, P. I., Ryu, J., Kim, Y. H., Chi, Y. T., (2010) Production of biosurfactant lipopeptides Iturin A, fengycin and surfactin A from Bacillus subtilis CMB32 for control of Colletotrichum gloeosporioides, *J Microbiol Biotechnol,* **20,** pp. 138-45.
[26] Ben Slimene, I., Tabbene, O., Djebali, N., Cosette, P., Schmitter, J. M., Jouenne, T., Urdaci, M. C., Limam, F., (2012) Putative use of a Bacillus subtilis L194 strain for biocontrol of Phoma medicaginis in Medicago truncatula seedlings, *Res Microbiol,* **163,** pp. 388-97.
[27] Mandal, S., Sharma, S., Pinnaka, A., Kumari, A., Korpole, S., (2013) Isolation and characterization of diverse antimicrobial lipopeptides produced by Citrobacter and Enterobacter, *BMC Microbiology,* **13,** pp. 152.
[28] Silva, M., Pereira, A., Teixeira, D., Candeias, A., Caldeira, A. T., (2016) Combined Use of NMR, LC-ESI-MS and Antifungal Tests for Rapid Detection of Bioactive Lipopeptides Produced by *Bacillus.*, *Advances in Microbiology,* **06,** pp. 788-796.

[29] Lopes Da Silva, Z., (2018). Avaliação da capacidade antifungica de extractos de folhas de Pouteria ramiflora sobre fungos leveduriformes e filamentos *PhD in Pharmaceutical Sciences*, University of Brasilia, Brasília.

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António Candeias is specialized in chemistry applied to cultural heritage and surface chemistry. He is Vice-rector for Research and Development and professor in the Chemistry Department of the University of Évora, Portugal. In terms of research activity, his main interests have focused on the study of mortars and renders, the study and characterization of cultural assets and archaeological materials and the optimization and application of analytical techniques for the study of cultural heritage, with special emphasis on micro-analysis and in-situ analysis.

Ana Teresa Caldeira is professor in the Department of Chemistry at the University of Évora, specialized in biochemical analysis and microbial physiology. She is the coordinator of the Biotechnology and Biodegradation Unit of the HERCULES Lab. Her main areas of research focus on new strategies for diagnosis, monitoring and mitigation applied to cultural heritage including new methods for measuring biodegradation, development of new green biocides and methodologies for the identification of biomolecules.

Summary

Damage to buildings and monuments by microbiological growth is a cause of serious concern. Due to the necessity of treatment being assessed for *in situ* application, it is important to select a safe and effective strategy approach that safeguards both

the environment and human beings. In the past decade the most frequently used and effective biocides have been banned due to their environmental and health hazards. This paper reports the development of remediation actions based on environmentally innocuous alternatives derived from active compounds produced by *Bacillus* sp. in conjugation with natural plant products from Brazilian Cerrado plant extracts.

Riassunto

I danni a edifici e monumenti, dovuti alla crescita microbiologica, sono motivo di grave preoccupazione. Per valutare il trattamento più efficace da applicare in situ, è importante individuare un approccio strategico, sicuro ed efficace che salvaguardi sia l'ambiente che gli esseri umani. Nell'ultimo decennio i biocidi più efficaci e frequentemente usati sono stati banditi a causa dei loro rischi ambientali e sanitari. Questo lavoro riporta lo sviluppo di azioni di bonifica basati su prodotti, innocui dal punto di vista ambientale, derivati da composti attivi del *Bacillus sp.* coniugati con estratti vegetali naturali di piante provenienti dal Cerrado brasiliano.