NATURAL BIOCIDES TO PREVENT THE MICROBIAL GROWTH ON CULTURAL HERITAGE

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Abstract

Many historic, cultural and artistic objects and buildings are made of stone. Like all materials, stone is subject to inexorable deterioration. Along with chemical and physical weathering factors, microbial growth plays an important role in this process. Stone types and local climatic differences have a great impact on the bio-deterioration process and on their outcomes.

Microbial metabolism products, organic and inorganic acid, chelating agents, enzymes and extracellular polymeric substances (EPS), are responsible of bio-corrosion and of bio-mineralization; furthermore phototropic and heterotrophic microorganisms (e.g.*Actinobacteria, Firmicutes* and fungi) are able to penetrate into stone mineral. In addition to structural damage, these microorganisms cause, also, aesthetic damage.

In general the biocides used to prevent the growth of microorganisms are mostly based by using chemical methods, but they show a restricted efficiency and can cause discolorations and severe damages to the cultural heritage and, also, they affect higher organisms including humans.

Recently, the biotechnology and the applied microbiology as the use of antagonistic organisms or their metabolites products against the bio-deteriorating agents, offers a wide range of new procedures for the cleaning and conservation of the artistic substrata. The aim of this work was to research and to develop new types of products that are not harmful to human health, with a low environmental impact, highly selective and at low cost. Secondary metabolites from Solanaceae extracts (glycoalkaloids), *Burkholderia gladioli* pv. *agaricicola* (*Bga*) ICMP 11096 strain and *Bga* cell-free filtrate were tested against a panel of microorganisms isolated from two bridges located in Potenza and in Campomaggiore (Southern of Italy). Artstone isolated bacteria belong to *Proteobacteria, Firmicutes* and *Actinobacteria*, while fungi belong to *Aspergillus*, *Penicillium, Coprinellus* and *Stemphylium* genera.

Glycoalkaloids, *Bga* and cell filtrate have inhibited the growth of microorganisms colonizing confirming that the application of bio-pesticides will be a promising alternative to synthetic pesticides. Moreover, use of these natural substances permits a more homogeneous removal of the surface deposits without compromising the substrata structure and preserves the patina noble.

Key words: bio-deterioration, secondary metabolites, bio cleaning.

Introduction

The science and technology interact with the art and culture in several ways: art is influenced by scientific discovery of new materials while the new materials science and the scientific examination of the artwork allows you to retrace the history of human civilization [Rinaldi, 2006]. Biotechnology can play a very important role in protecting and preserving the cultural heritage for future generations. The use of molecular techniques in the study of the phenomena of bio-decay represents a new and valuable tool for the diagnosis and preventive conservation. This has allowed in a few years to greatly expand the knowledge on the complex interactions between microorganisms and the cultural heritages with the aim to understand the role that individual organisms play within the microbial community and in the processes of bio-deterioration and the bio-remediation together.

Microbial metabolism produces deteriorating agents such as organic and inorganic acid, chelating agents, enzymes and extracellular polymeric substances (EPS) causing e.g. bio-corrosion and bio-mineralization; furthermore phototropic and heterotropic microorganisms (e.g.*Actinobacteria, Firmicutes* and fungi) are able to penetrate into stone mineral [Suihko et al., 2007, Warscheid and Braams, 2000, Morton and Surman, 1994]. In addition to structural damage, these microorganisms cause, also, aesthetic damage.

Microorganisms are generally associated to detrimental effects on stone [Ranalli et al.,2003, Sanchez-Moral et al, 2004]. However, it has been seen that they can be used for the removal of harmful compounds on

artistic objects [Gauri, et al., 1992; Ranalli et al., 1997, 2005]. Gauri et al. have used *Desulfovibrio desulfuricans* for the cleaning of an old gypsum-encrusted marble statue that was previously consolidated. Some researchers have compared two cleaning methods: traditional (ammonium carbonate-EDTA mixture) and innovative (sulfate-reducing bacterium *Desulfovibrio vulgaris* subsp. vulgaris ATCC 29579) for the removal of black crust (containing gypsum) on marble of the Milan Cathedral (Italy) [Cappitelli et al., 2007]. Biological cleaning has showed an homogeneous removal of the surface deposits and has preserved the patina noble under the black crust. Whereas both of the treatments converted gypsum to calcite, allowing consolidation, the chemical treatment also formed undesirable sodium sulfate.

The goal of this work was to research and to test new types of bio-cleaning products that are not harmful to human health, with a low environmental impact, highly selective and at low cost: the secondary metabolites.

These bio-chemicals are produced by microorganisms and plants with differents functions and they can be classified on the basis of chemical structure, composition, solubility in various solvents, etc.

In this research was studied the activity of secondary metabolites of *Burkholderia gladioli* pv. *agaricicola* and of glycoalkaloids.

Burkholderia gladioli, aerobic gram-negative rod-shaped bacterium, has the ability to produce in vitro secondary metabolites with relevant biological activities that may have potential practical applications [Elshafie et al., 2012].

Glycoalkaloids are important bioactive secondary metabolites commonly found in Solanaceae plants. They have anti-bacterial and anti-fungal activity [Ventrella et al., 2012].

Secondary metabolites from *Burkholderia gladioli* pv. *agaricicola* (*Bga*) ICMP 11096 strain and *Bga* cell-free filtrate and glycoalkaloids extracts from Solanaceae were tested against a panel of microorganisms isolated from two bridges located in Potenza and in Campomaggiore (Southern of Italy) (Fig.1).

Fig.1: San Vito bridge in Potenza (a) and Della Vecchia bridge in Campomaggiore (b)

Materials and Methods

Sampling, isolation and growth conditions. 31 samples of two bridges (22 of San Vito bridge and 9 of Della Vecchia bridge) were taken by carefully scraping off material with sterile swabs and scalpel in according to the *Italian Cultural Heritage Ministry Recommendation 3/80* and re-suspended in saline solution buffer(0.85% NaCl). Samples were collected in duplicate and isolated by spread plating on PCA medium. For each sample, different colonies were selected and purified by streaking on PCA added with tetracycline hydrochloride (0.005 g/l) for fungi growth and on PCA added with cicloxiamide (70/100ppm) for bacteria growth. Colonies were used to test morphology, Gram reaction and catalase. Isolates were routinely cultivated in PCB and maintained frozen (-80°C) in skim milk.

Molecular identification

DNA extraction

As Bacterial isolates, the total DNA was extracted by using the *Marmur* method *(1961)* modified; while as fungal isolates, the total DNA was extracted by using the Raeder & Broda method (1985) modified. 25 ng of DNA were used for PCR amplification.

16S rDNA amplification and sequencing

Synthetic oligonucleotide primers fD1 (AGAGTTTGATCCTGGCTCAG) and rD1 (AAGGAGGTGATCCAGCC), were used to amplify the 16S rDNA. PCR mixture and PCR amplification conditions was performed as previously reported [Bonomo et al., 2008]. The PCR products were sequenced and DNA similarity was performed with the Gene Bank and EMBL database. The Gene Bank accession numbers of the sequences are reported in Table 1.

Internal transcribed spacer (ITS) region amplification

As described previously [White et al., 1990], primers ITS1 and ITS4 were used to amplify specific ITS regions of fungal ribosomal genes. PCR mixture and amplification conditions were performed as described by White et al. [1990]. The PCR products were sequenced and DNA similarity was performed with the Gene Bank and EMBL database.

Antibacterial assay

Burkholderia gladioli pv. *agaricicola* ICMP11096 (*Bga*), obtained from International Collection of Microorganisms from Plant (ICMP), was used as reference strain and grown in King Agar B (KB) medium for 24 h at 30°C.

The antibacterial activity of *Bga* and cell-free filtrates of *Bga* was tested against all bacterial isolates by agar well diffusion method. The cell-free filtrate of *Bga* was obtained by inoculum of 150 ml of liquid minimal mineral medium (MM) with 1.5 ml of bacterial suspension and, after incubation at 30°C for 5 days, the culture was filtered (Millipore, 0.20 µm) [Elshafie et al., 2012].

Moreover, the antimicrobial activity of glycoalkaloids was also evaluated. Glycoalkaloids were obtained by unripe berries of *Solanum nigrum* (European Black Nightshade) and extracted by method of Cataldi et al. [2005]. The extract was lyophilized and re-suspended in water to obtain the stock solution of solamargine (principal component) at concentration of 500 µM. The agar media were inoculated with 60 µl of glycoalkaloids, *Bga* and cell-free filtrates of *Bga* and after incubation for 3 days at 30°C, the inhibition zone diameter was measured in cm.

Antifungal assay

The antibacterial activity of *Bga* and cell-free filtrates of *Bga* was evaluated by diffusion method. *Bga* or cell-free filtrate of *Bga* was inoculated in PCA plates containing 1 cm^2 of fungal disc. After 4-5 days of incubation at 30°C, the diameter of fungal colonies were scored and measured in cm.

The fungitoxicity was expressed as percentage of growth inhibition (PGI) and calculated according to Zygaldo et al. [1994] formula:

$$PGI\ (\%) = 100(Gc-Gt)/Gc$$

where Gc represents the average diameter of fungi grown in PCA (control); Gt represents the average diameter of fungi cultivated on the treated PCA dish containing the antagonistic bacteria.

Results and Discussion

On the basis of amplification and partial sequencing of 16S rDNA, the bacterial strains were grouped in 21 different species belonged to genera within *Proteobacteria*, *Firmicutes* and *Actinobacteria* phyla. Table 1 describes the bacterial strains studied, the molecular identification and the Gene Bank accession numbers of sequences.

Tab.1. Molecular identification of bacterial strains isolated from two brigdes

Strains	Source	Identified as [a]	Accession numbers
YIM6	Della Vecchia bridge	*Streptomyces beijangensis* (95%)	NR_028825.1
CF17	Della Vecchia bridge	*Rhodococcus fascians (*96%)	NR_037021.1
R551-3	Della Vecchia bridge	*Stenotrophomonas maltophilia* (90%)	NR_074875.1
GTC1228	Della Vecchia bridge	*Staphilococcus hominis subsp.novobiosepticus*(84%)	NR_041323.1
DSM20416	Della Vecchia bridge	*Exiguobacterium acetylicum* (96%)	NR_043479.1
255-15	Della Vecchia bridge	*Exiguobacterium sibiricum* (89%)	NR_075006.1
DSM 1321	Della Vecchia bridge	*Bacillus simplex* (93%)	NR_042136.1
G2-1	Della Vecchia bridge	*Arthrobacter nitroguajacolicus* (94%)	NR_027199.1
Ames	San Vito bridge	*Bacillus anthracis* (91%)	NR_074453.1
GC 65	San Vito bridge	*Bacillus cereus* (98%)	KF158234.1
DSM 2055	San Vito bridge	*Arthobacter agilis* (97%)	NR_026198.1
R5812	San Vito bridge	*Arthrobacter grandavensis* (98%)	NR_025475.1
DSM20119	San Vito bridge	*Arthrobacter oxydans* (92%)	NR_026236.1
DSM2898	San Vito bridge	*Lysinibacillus fusiformis* (87%)	NR_042072.1
GTC843	San Vito bridge	*Staphilococcus saprophiticus subsp.bovis* (96%)	NR_041324.1
AW25	San Vito bridge	*Staphilococcus warneri* (98%)	NR_025922.1
CFML 96-170	San Vito bridge	*Pseudomonas orientalis (94%)*	NR_024909.1
5-1-1	San Vito bridge	*Planococcus psychrotoleratus (97%)*	GU113006.1
DSM14481	San Vito bridge	*Exiguobacterium undae* (98%)	NR_043477.1
JCM9073	San Vito bridge	*Paenibacillus lautus (94%)*	NR_040882.1
P333/02	San Vito bridge	*Microbacterium foliorum* (98%)	NR_025368.1

[a] % of similarity on the basis of partial sequencing of 16S rDNA

The fungi isolated on two bridges belonged to *Aspergillus*, *Penicillium, Fusarium, Coprinellus* and *Stemphylium* genera.

Inhibition activity

The ability of *Bga*, cell-free filtrate of *Bga* and glycoalkaloid extracts to inhibit the growth of bacteria and fungi isolated on two bridges was evaluated in this study.

Results showed that glycoalkaloids extracts were able to inhibit the growth of all bacterial isolates while *Bga* showed an high inhibition ability against *Planococcus psychrotoleratus* growth.

Cell-free filtrate of *Bga* inhibited the growth of *Planococcus psychrotoleratus, Exiguobacterium undae, Exiguobacterium acetylicum, Exiguobacterium sibiricum* and *Bacillus cereus* (fig.2).

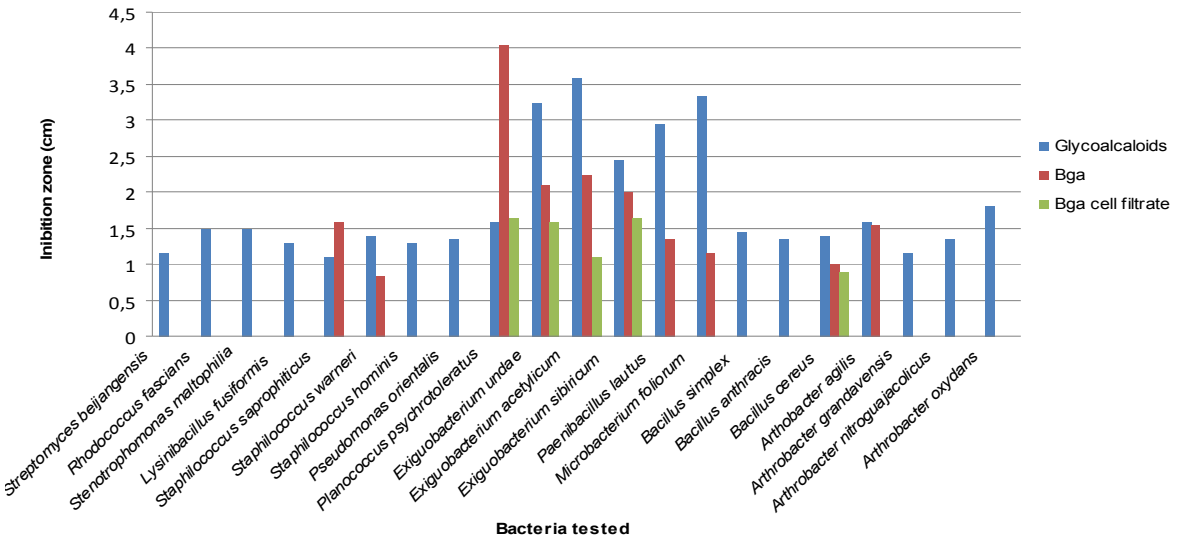

Fig.2.: Antibacterial activity of glycoalkaloids, *Bga* and cell-free filtrate of *Bga*

As shown in Figure 3, *Bga* proved an higher antifungal activity than the cell-free filtrate of *Bga* The Highest percentage of inhibition of *Bga* against fungal growth was observed versus *Penicillium* spp. (75%). The inhibition scale was Penicillium > *Stemphylium vesicarium*> *Coprinellus*> *Aspergillus*. The cell-free filtrate of *Bga* was, generally, less active.

Solanaceae extracts tested against *Fusarium* genus showed a low activity confirming results obtained by other researcher [Ventrella et al, 2012]. Tests against the other fungal colonies, founded on the bridges, are in progress.

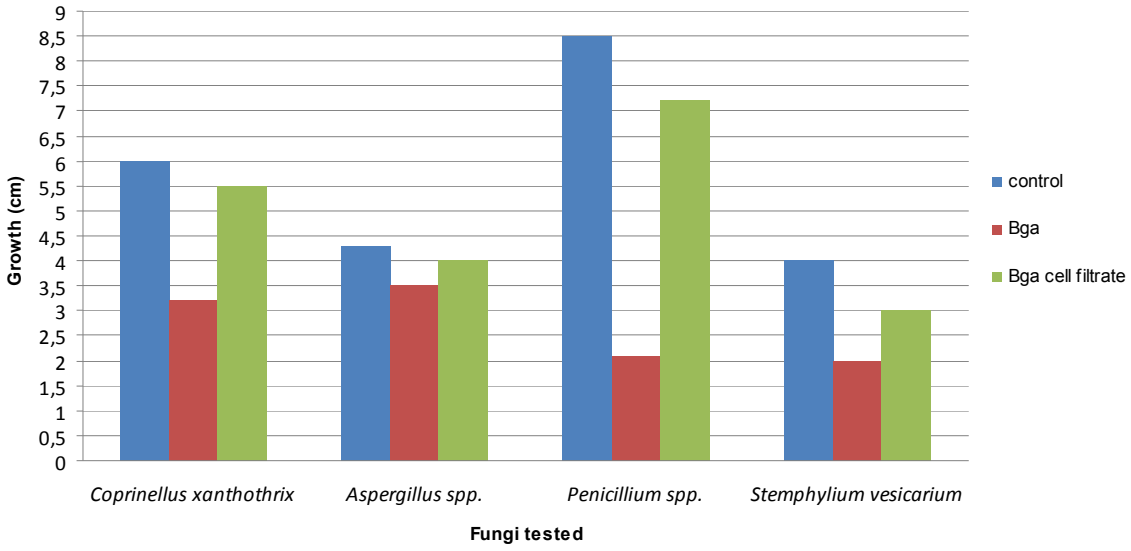

Fig.3: Antifungal activity of *Bga* and cell-free filtrate of *Bga*

Conclusion

The coexistence of bacterial and fungal species on the stones means that the microorganisms interact each other through a series of complex mechanisms. Their presence can cause biodeterioration phenomena on the stone surfaces due to the formation of patinas and biofilms in which either phototropic microorganisms or chemorganotrophic bacteria could prevail.

Glycoalkaloids extracts has inhibited all bacterial strains tested, while the Bga broth and the cell-free filtrate resulted more selective especially against bacteria belonging to Firmicutes phylum. Antifungal activity was less obvious: probably, this is due to the structural complexity of fungi.

The high activity of glycoalkaloids has confirmed results obtained by other researchers that have tested, only in the agricultural domain, the repulsive or toxic effects of theses substances on some insects such as the coleoptera Leptinotarsa decemlineata and Agriotes obscurus [Jonasson and Olsson, 1994; Sinden et al. 1980, 1986]. Marciniak P. et al, 2009, has reported that the application of glycoalkaloids on the continuously perfused Z. atratus heart has inhibited progressively frequency contractions; higher concentrations exerted short and reversible cardiac arrests.

The application of glycoalkaloids and metabolites of Bulkolderia on cultural heritage would be an innovative challenge and may be a viable alternative to synthetic pesticides for preservation of cultural heritage and for the safety of human health and the environment.

The use of these innovative tools can favour the maintenance of the dynamic ecosystem equilibrium allowing the implementation of non invasive treatment methods.

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