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TOXICOLOGICAL ASSESSMENT OF NOVEL GREEN BIOCIDES FOR CULTURAL HERITAGE

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Abstract

The damaging of buildings and monuments by biological contamination is a cause of serious concern. Biocides based on chemical toxic compounds have been used to mitigate this problem. However, in the past decade many of the most effective biocides have been banned due to their environmental and health hazards. Therefore, proper remediation actions for microbiologically contaminated historic materials based on environmentally safe solution is of vital importance. Bacillus species are emerging as a promising alternative for built heritage treatment. They produce a great diversity of secondary metabolites with biological activity, well known to possess antagonistic activities against many fungal pathogens. In order to evaluate the antifungal activity of the novel biocides produced in our laboratory by cultures of selected bacterial strains, liquid interaction assays using four biodeteriogenic fungi were achieved, revealing a nearly 100% of inhibitory capacity to fungal proliferation. To confirm their effective safe toxicological properties, in vivo tests using two different biological models were performed. The lyophilized supernatant of the Bacillus culture broth showed no lethality against brine shrimp and also no toxicological effects in Swiss mice through administration of acute dose of 5000 mg/kg by oral gavage. In fact, the bioactive compounds were no lethal at the tested dose unlike Preventol® (commercial biocide) that induced acute toxicity with 10 times minor concentration dose administrated in the same conditions. Therefore, the new bioactive compounds that suppress growth of biodeteriogenic fungi on historical artworks, presenting at the same time no toxicity against other living organisms, constituting an efficient and green safe solution for biodegradation/biodeterioration treatment of Cultural Heritage.

Keywords: *Bacillus sp.; Biodegradation/biodeterioration; Bioactive compounds; Biocides; Toxicity*

Introduction

The damaging of buildings and monuments by biological contamination is a cause of serious concern. Biological growths are a potential cause of long term decay, caused by physical and chemical damage of building materials [1]. The prevention of microorganism colonization in Cultural Heritage assets as well as the development of appropriate treatment

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measures for contaminated objects is a challenge for restorers, museum curators and architects [2, 3]. This has implications for the techniques of cleaning and conservation of objects but also consequences for the occupational safety and health of restorers. Biocides based on chemical toxic compounds have been used to mitigate these problem. However, in the past decade many of the most effective biocides have been banned due to their environmental and health hazards [4].

Many authors have used several products having biocide characteristics, in order to eliminate the biodeteriogenic organisms that have developed on stone monuments or rock sites [5-7]. These products are commercially available both as active principle or formulates and cover a wide range of chemical classes, from very simple inorganic compounds such as Na and Ca hypochlorite to very complex organic ones such as the Quaternary Ammonium Compounds [8, 9]. However, the chemical biocide commonly used, act through toxic mechanism exhibiting numerous pharmacological activities toward a number of specific cellular targets, including damaging or inhibiting the synthesis of cell walls and affecting DNA or RNA, proteins or metabolic pathways [10]. Therefore, developing proper remediation actions for microbiologically contaminated historic materials based on environmentally safe solution is of vital importance.

Bacillus species are emerging as a promising alternative for built heritage treatment and rehabilitation due to its capacity to produce a great diversity of secondary metabolites with biological activity. In fact, some strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been referred to produce antifungal lipopeptides [11-13] such as surfactin, fengycin and iturin. These compounds act as amphiphilic membrane-active biosurfactants with potent antagonistic activities against many fungal pathogens [14]. Due to their low molecular weight, this lipopeptides are capable of changing the physical and chemical properties of interfaces, increasing the surface area from non-soluble hydrophobic growth substrates and the solubility of hydrophobic substances, which have interference on the microorganism's adherence and detachment from surfaces [1]. Clinical trials on humans and animals have also shown iturin A to be a valuable drug due to its broad antifungal spectrum, low toxicity and low allergic effect [15-17]. The nontoxic mechanism of action of these amphiphilic cyclic biosurfactants are therefore directly related with unique features, high biodegradability, non-harmful and environmentally friendly characteristics.

For that reason, the present paper was envisaged to evaluate the effective safe toxicological properties of the new bioactive compounds produced in our laboratory by *Bacillus* sp. liquid cultures using two different biological models. Simultaneously, the potential antifungal capacity was tested against biodeteriogenic fungal strains isolated from biodegraded Cultural Heritage assets in order to clarify the effectiveness of this new alternative to the production of novel green biocides.

Materials and Methods

Microorganisms and culture conditions

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) [11]. Cells were maintained on Nutrient Agar (HIMEDIA) slants and stored at 4ºC. The fungal cultures were maintained on malt extract agar (MEA, HIMEDIA) slants and used as test microorganisms. The strains *Penicillium* sp., *Alternaria* sp., *Mucor* sp. and *Fusarium oxysporum* were isolated from biodegraded mural paintings and belonging to the laboratory collection (HERCULES-Biotec laboratory, Évora University).

Bioactive compounds production

The *Bacillus* sp. CCMI 1051, CCMI 1052 and CCMI 1053 cells were inoculated in 100 mL of Nutrient Broth (HIMEDIA) medium. The liquid cultures were incubated for 72 hours at 30ºC in an orbital shaker at 150 rpm (IKA KS 4000 I control). After 48 hours (stationary-phase) of culture growth, the bacterial cells were removed from the culture by centrifugation (1,000 × g for 10min at 4ºC) fouled by membrane filtration (0,2 μm) and the supernatant, culture broth (CB) maintained at -20ºC for further analysis. The new active compounds produced by Bacillus CCMI 1051, CCMI 1052 and CCMI 1053 strains was quantified by LC-ESI-MS (LCQ Advantage ThermoFinnigan mass spectrometer, Zorbax Eclipse, C18 column) and will be designated by CB1, CB2 and CB3, respectively.

Antifungal assay

In order to evaluate the antifungal activity of the biocides produced a diffusion disk assays and an interaction in liquid medium assay were performed [18]. A fungal spore suspension of biodeteriogenic fungal strain in test was prepared suspending loopfuls of hyphae and spores in 5mL of 0.9% NaCl solution. The spore suspension was obtained through a serial dilution and adjusted to 10^5CFU/mL. A mixture composed by 5mL of malt extract, 0.5mL of 10^5CFU/mL of spore suspension and 5mL of CB was incubated at 28ºC for 24h using an orbital shaker at 125rpm. One millilitre of each interaction mixture was plated, by incorporation in 20mL of Cook Rose Bengal agar (HIMEDIA), and the Petri dishes were incubated at 28ºC for 24–48h. In the antifungal interaction assay, the relative inhibition growth were determined by counting the number of CFU (colony forming units) against a blank test (%) of the same fungus in the absence of the *Bacillus* sp. supernatant [11, 19].

The Fungal spore suspensions prepared were also incorporated in MEA at 45ºC in Petri dishes. Filter paper discs (Macherey-Nagel 827 ATD) impregnated with 20μL of *Bacillus* CB, after cells removal were placed on the agar and the Petri dishes were incubated at 25ºC for 24–48h. In the disc diffusion assay the inhibition level were determined by measure the diameter of the inhibition halo around the paper disc in the presence of bioactive compounds produced [20].

Acute toxicity assessment

Toxicity in Artemia salina

The toxicity of the lyophilized supernatant of the *Bacillus* CB and three commercial chemical biocides Preventol® (2-Phenylphenol, Lanxess, Leverkusen, Germany), NEW DES® (4-(2-phenylethoxy)-quinazoline, Helios Group, Vicenza, Italy) and Panacide® (Dichlorophen, BCM, Hillsborough, USA) was evaluated using *Artemia salina* test kit (Artoxkit MTM, Microbiotest). All the compounds were tested in a range of concentrations of 1000-0.1μg/mL in order to stablish lethal concentrations (LC_{50}). A solution of sea water (Artoxkit MTM, Microbiotest) was used as negative control and the potassium dichromate (100 mg/ mL) as a positive control [21].

Acute toxicity in Swiss mice

The acute toxicity assay was performed using *Swiss* mice (*Mus musculus*). The LD_{50} was evaluated through the up and down protocol (OECD 425), according to the guidelines of the Organization for Economic Co-operation and Development [22].

Male albine *Swiss* mice (40 ± 5g), with two months of age, were used. The animals were randomly divided into five groups, each one with three animals and were fasted 24 hours before testing, with water *ad libitum*. Samples of CBs and Preventol® biocide were orally administered, with the aid of a gastric probe, at a concentration of 5000 mg/kg for the supernatant and 200 to 1000mg/kg for Preventol® D 7, using distilled water as the vehicle. Group 1, the negative control, was administrated only with the vehicle (distilled water). Group 2, 3 and 4 were administrated with CB1, CB2 and CB3, respectively. The group 5 was administered with 2-Phenylphenol referred to throughout as Preventol® D 7, acting as a positive control group (Fig. 1).

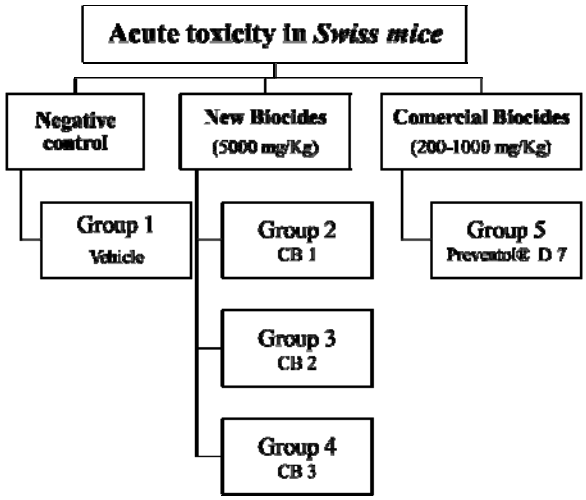

Fig. 1. Schematic representation of the acute toxicity assay on *Swiss mice*.

Additionally, a pharmacological screening was performed in order to observe the behaviour and activity of the animals at 0, 1, 2, 4, 6 and 24h after oral administration. Tests were based on: reflexes (pineal, corneal, posture, ipsilateral anterior and posterior), motor activity (catalepsy, traction) and behaviour observation (aggression, passivity and fear).

The animals were kept under observation for 15 days [22], and during these time were acclimated under controlled temperature (23 ± 1°C), 12h light/12h dark, during which time they had free access to food and water *ad libitum* [23, 24].

All the experimental animals' procedures were following by a Competent Researcher, according the Recommendations of the General Direction of Veterinary (Order 1005/92, October 23) and the Federation of European Laboratory Animal Science Associations (FELASA) (nº 020/08).

Results and discussions

Antifungal activity of bioactive compounds

The supernatants of *Bacillus* sp. liquid cultures were tested against the biodeteriogenic fungal strains isolated from biodegraded mural paintings: *Penicillium* sp., *Alternaria* sp., *Mucor* sp. and *Fusarium oxysporum* The results of the antifungal activity assays show a higher inhibited capacity against *Penicillium* sp. mainly with the bioactive compounds from CB 2 and CB 3 (Fig. 2). *Mucor* sp. was the less inhibited fungi, however their inhibition release 76% when in interaction with supernatants of *Bacillus* sp. CCMI 1051 cultures (Fig. 3).

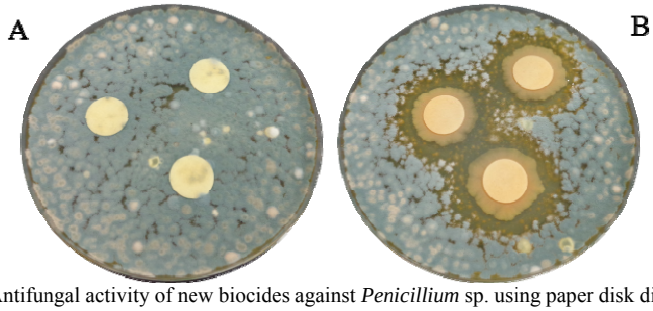

Fig. 2. Antifungal activity of new biocides against *Penicillium* sp. using paper disk diffusion assay: A- Control; B- In the presence of 10µL of supernatant of culture after cells removal.

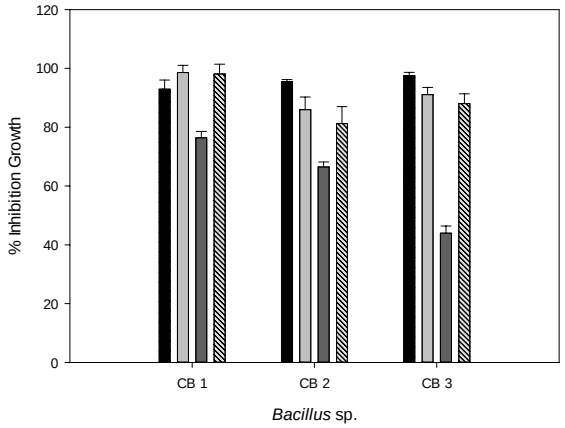

Fig. 3. % of inhibition growth resulted from interaction assays between biodeteriogenic fungi and *Bacillus* sp. cell-free CB. *Penicillium* sp.; *Alternaria* sp.; *Mucor* sp.; *Fusarium oxysporum*

Particularly high antagonistic activity nearly to 100% was detected for all bacillus cell-free supernatant of cultures, against *Fusarium oxysporium*, *Penicillium* sp. and *Alternaria* sp. The heritage biodeteriogenic fungi tested can be inhibited in the presence of the new compounds produced by bacterial cultures, showing the great potential of this biotechnological approach applied to heritage context.

Toxicological evaluation

The toxicity of the bioactive compounds in the lyophilized supernatant of *Bacillus* strains CB were evaluated *in vivo* using two different biological models. The *brine shrimp Artemia salina* was used as a standard test for determination of LC_{50} and a test of acute toxicity was performed in *Swiss* mice for the determination of LD_{50} (OECD guidelines) [22].

For *Artemia salina* model the lyophilized supernatant of the *Bacillus* CB showed a low toxicity with mortality values less than 3,5% at a concentration of 1000 µg/mL (Fig.4). The negative control, seawater, caused no mortality and potassium dichromate ($K_2Cr_2O_7$) caused 95.5% of mortality at 100mg/mL.

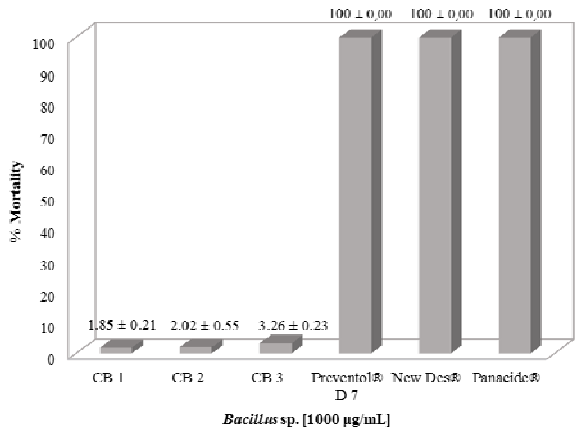

Fig. 4. % of *Artemia salina* mortality for bioactive compounds produced and commercial biocides.

The three commercial chemical biocides Preventol® D 7, NEW DES® and Panacide® were tested in the same conditions in order to access and compare the level of toxicity with the natural bioactive compounds produced (Table 1). This assay allows to evaluate the potential of lethal effect in a living organisms and also provides the basis for further analysis with more representative biological models. The results reveal that Preventol® D 7 cause 50% of mortality with a concentration of 5.07 ± 0.35µg/mL, showing a higher toxicity than NEW DES® (34.53 ± 4.70µg/mL) and Panacide® (LC_{50} of 257.48 ± 8.54µg/mL). Therefore, the toxicity of the bioactive compounds produced was evaluated in mammals using Preventol® D 7 as positive control and *Swiss* mice as model. CB1, CB2 and CB3 did not show lethality according to OECD guidelines [22], with LD_{50} values greater than 5000mg/Kg. Additionally, pharmacological screening was conducted in *Swiss* mice, based on tests reflexes (pineal, corneal, posture, ipsilateral anterior and posterior), tests of motor activity (catalepsy, traction) and observation of behavior (aggression, passivity and fear). The animals showed normal motor, cognitive and sensorial behavior during the first 24 hours of the assay, revealing also no signs of toxicity. In the other hand, Preventol® D 7 used as positive control, evidence the death of all animals in the group treated with 1000 and 500mg/Kg. However, the same result do not occur with the group administrated with 200mg/Kg, where the animals showed a normal motor, cognitive and sensorial behavior. Consequently, the LD_{50} value of Preventol® D 7 is set between 200 and 500mg/mL.

Table 1. LC_{50} of biocides tested.

Biocide	LC_{50} (µg/mL)
Preventol® D 7	5.07 ± 0.35
NEW DES®	34.53 ± 4.70
Panacide®	257.48 ± 8.54
CB 1	>>1000
CB 2	>>1000
CB 3	>>1000

Thus, the new bioactive compounds produced do not induce acute toxicity, motor, cognitive or sensorial alterations at the tested dose, unlike Preventol® D 7 induced lethality when administrated with a dose 10 times lower.

Conclusions

The new biocides produced by *Bacillus sp.* cultures are bioactive compounds which has a great potential to suppress biodeteriogenic fungi growth on historical artworks. The absence of toxicity against living organisms, namely mammals, constitute an efficient and green safe promising alternative for biodegradation/biodeterioration treatment of Cultural Heritage.

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