

Combined Use of NMR, LC-ESI-MS and Antifungal Tests for Rapid Detection of Bioactive Lipopeptides Produced by *Bacillus*

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Abstract

The science and technology interact with the art in several ways. Biotechnological coupled with analytical approaches can play an important role in protecting and preserving cultural heritage for future generations. Many microorganisms influenced by environmental conditions are the main responsible for biological contamination in built heritage. Biocides based on chemical compounds have been used to mitigate this problem. Thus, it is vitally important to develop proper remediation actions based on environmentally innocuous alternative. *Bacillus* specie is emerging as an optimistic alternative for built heritage treatment due to their capacity to produce secondary metabolites with antagonistic activities against many fungal pathogens. Therefore, the intent of this work was to access a rapid evaluation of antifungal potential of bioactive metabolites produced by *Bacillus* strains and simultaneously their characterization using spectroscopic (NMR) and chromatographic techniques (LC-ESI-MS). The high antifungal activity obtained for *Bacillus* sp. active compounds produced in this study confirms the great potential to suppress biodeteriogenic fungi growth on historical artworks. Additionally, the proposed methodology allowed to access bioactive metabolites produced without need of the laborious total previous isolation and could be used as a viable alternative to be employed for screening and production of new green biocides.

Keywords

Lipopeptides, *Bacillus* sp., Biodegradation/Biodeterioration, Natural Biocides, Nuclear Magnetic Resonance, Liquid Chromatography Coupled to Mass Spectrometry

1. Introduction

The problems caused by lack of proper preservation of historical built heritage often only come to people's attention when a tragedy occurs such as a fire or collapse. Far from the eyes of most, silence threats lurking permanently our cultural heritage. Microorganisms, including bacteria, fungi, algae and lichens, influenced by environmental conditions are the main biodeteriogens agents responsible for esthetical damage and structural damage [1]. Colonization of heritage structures by fungi of the genera *Penicillium*, *Cladosporium*, *Alternaria*, *Curvularia*, *Dreschlera*, *Chaetomium*, *Fusarium*, *Trichoderma*, *Gliomastix*, *Aureobasidium* and *Mucor* can induce discoloration and deterioration of its surface, leading to the appearance of stains that alter the color of painted layer [2]-[4].

The procedure of controlling biodegradation/biodeterioration of artworks with minimal environmental impact it is a major challenge for the professionals responsible for the conservation and preservation of built heritage [5]. The biocides commonly used in a restoration/conservation intervention are chemical biocides like inorganic compounds (Na and Ca hypochlorite) and very complex organic ones such as the Quaternary Ammonium Compounds (Preventol R50, Neo-Desogen) that constitute a serious environmental and human health issue that is urgent to solve [6]. The solution may be closer than ever.

Bacillus species produce a great diversity of secondary metabolites with biological activity [3] [5], namely they are well known to possess antagonistic activities against many fungal pathogens [7]. These strains, in response to nutritional stress, activate a variety of processes including sporulation, synthesis of extracellular degradative enzymes and antibiotic production [8].

Some strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been referred to produce antifungal peptides [9]-[11]. The lipopeptides surfactin, fengycin and iturin are amphiphilic membrane-active biosurfactants with potent antifungal activities [12] which can be used as additive in new harmful and environmental friendly biocides.

The aim of this study was to access a rapid evaluation of antifungal potential of bioactive metabolites produced by *Bacillus* strains and simultaneously their characterization using NMR and LC-ESI-MS analysis without need of previous isolation.

2. Materials and Methods

2.1. Microorganisms and Growth Conditions

The strains of *Bacillus* sp. CCMI 1051, CCMI 1052 and CCMI 1053 were isolated from healthy *Quercussuber* 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) [9]. *Bacillus* 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 *Mucor* sp., *Alternaria* sp., *Penicillium* sp. and *Cl-*

dosporium sp., belonging to the laboratory collection (HERCULES-Biotec laboratory, Évora University), were isolated from biodegraded mural paintings, being also associated with known pathologies [4] [13].

2.2. Bioactive Compounds Production

For the production of bioactive compounds *Bacillus* sp. CCMI 1051, CCMI 1052 and CCMI 1053 cells were cultivated in 2 L of Nutrient Broth (HIMEDIA) medium and incubated for 48 hours at 30°C 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 broth by centrifugation (10,000× g for 10 min at 4°C). The supernatant were stored at –80°C and lyophilized for further analysis.

2.3. Column Chromatography

Antimicrobial compounds in lyophilized cell-free supernatant cultures were solubilized in CHCl₃/CH₃OH/H₂O (65:35:5) and purified by flash column chromatography on silica gel (Merck, Kieselgel 60 with 0.040 - 0.063 mm) using the system CHCl₃/CH₃OH/H₂O (65:35:5) as eluent. The bioactive compounds purification was monitored by thin layer chromatography (Merck, Kieselgel GF 254, 0.2 mm) using the same eluent.

2.4. Antifungal Assay

Fungal spore suspension of *Mucor* sp., *Alternaria* sp., *Penicillium* sp. and *Cladosporium* sp. were prepared by adding loopful of hyphae and spores from a Malt Extract Agar (MEA) slant incubated at 25°C for 7 days, in 15 mL of NaCl 0.85% solution. The suspension was filtered by sterilized cotton or triple gauze. A 10⁶ 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) with 13 millimeters of diameter impregnated with 20 µL of *Bacillus* culture broth after cells removed were placed on the agar. The Petri dishes were incubated at 25°C for 48 - 72 h. Antifungal activity was indicated by the formation of an inhibition halos around the discs.

2.5. LC-ESI-MS Analysis

Before and after compounds separation, the different fractions obtained was filtered with a 0.45 µm nylon filter (VWR International, West Chester, PA, USA), and a 10 µL sample was analysed by liquid chromatography coupled to mass spectrometry (LC-MS).

LC-ESI-MS analyses were carried out in a LCQ Advantage Thermo Finnigan mass spectrometer equipped with an electrospray ionization (ESI) source and using an ion trap mass analyzer. The conditions of lipopeptides analysis were: capillary temperature 300 °C, source voltage 5.0 kV, source current 100.0 A, and capillary voltage 22 V, in positive mode. The mass spectrometer equipment was coupled to an HPLC system with

autosampler (Surveyor ThermoFinnigan). The analytical column was a reversed phase Zorbax Eclipse (C18, particle size 5.0 μm , 150 mm \times 2.4 mm). The chromatographic separation was performed with a gradient program using acetonitrile as eluent A and water acidified with 0.1% (v/v) formic acid as eluent B, at a flow rate of 0.3 mL \cdot min $^{-1}$. The elution program was as following: linear gradient from 20% to 50% of A (0 - 10 min) and from 50% to 100% of A (10 - 40 min).

2.6. Nuclear Magnetic Resonance (NMR)

^1H NMR spectra were recorded on a Bruker Avance III HD 400 spectrometer at 400 MHz in DMSO- d_6 (Euriso-top). ^1H shifts are reported relative to the ^1H signal of DMSO- d_6 ($\delta = 2.50$ ppm) [14] [15].

3. Results and Discussion

3.1. Antifungal Activity Assessment

Several studies have described that bacteria of genera *Bacillus* are worthy to be used as antifungal substances producer including extracellular lipopeptides for versatile applications [16]-[19]. To detect these biosurfactants several qualitative tests are available [20].

In this work a new methodological scheme using analytical and biological approaches was established in order to access bioactive metabolites produced by *Bacillus* strains and simultaneously their characterization without need of the laborious total previous isolation.

The final propose is the production of a new alternative for commercial toxic biocides used in heritage context thought the development of green biocides produced by harmful *Bacillus* species.

After 48 h of *Bacillus* sp. CCMI 1053 culture, the supernatants of liquid cultures were tested against the selected biodeteriogenic fungal strains isolated from biodegraded mural paintings: *Mucor* sp., *Alternaria* sp., *Penicillium* sp. and *Cladosporium* sp. (Table 1). These fungal strains represent different fungi genera commonly found in biodegraded heritage materials and their inhibition is an important issue in this context.

Antifungal assays show a high inhibition zone for all free cells culture broths produced against biodeteriogenic fungi. Nevertheless the *Bacillus* sp. CCMI1053 display a major antifungal potential against *Penicillium* sp., confirmed by the formation of an inhibition halo of 37.44 mm (Figure 1 and Table 1). Results obtained in this work

Table 1. Inhibition halos of *Bacillus* sp. against the biodeteriogenic fungi.

<i>Bacillus</i> sp.	Inhibition halos (mm)			
	<i>Mucor</i> sp.	<i>Alternaria</i> sp.	<i>Penicillium</i> sp.	<i>Cladosporium</i> sp.
CCMI 1051	25.67 \pm 1.00	23.11 \pm 0.93	23.67 \pm 0.50	22.11 \pm 0.19
CCMI 1052	18.22 \pm 0.97	17.89 \pm 0.33	18.89 \pm 1.05	17.33 \pm 0.03
CCMI 1053	27.33 \pm 0.87	22.33 \pm 0.71	37.44 \pm 1.83	26.67 \pm 1.41

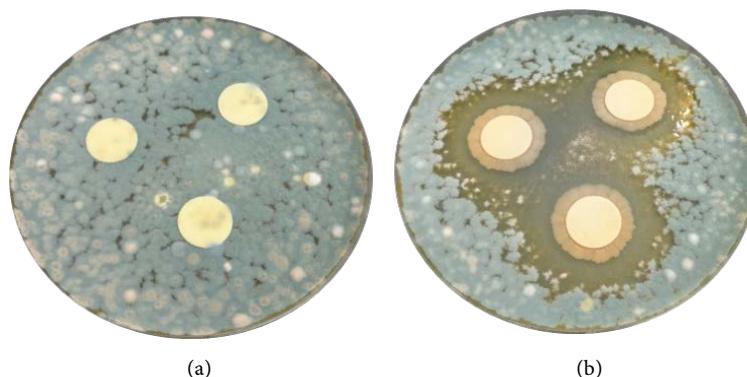


Figure 1. Antifungal activity of biocides against *Penicillium* sp. using paper disk diffusion assay. (a) Control; (b) In the presence of *Bacillus* sp. CCMI 1053 cell free culture Broth.

accomplish a ground-breaking research for the utilization of the compounds produced for application in heritage materials contaminated by fungal communities and confirm previous studies reporting that *Bacillus* strains have a height antifungal activity [10] [17] [21] [22] related to the presence of lipopeptides.

3.2. Characterization of the Antifungal Compounds

The $^1\text{H-NMR}$ spectrum of the bioactive compounds showed signals for N-binding protons at δ 6.8 - 8.6 ppm, α -protons at δ 4.0 - 4.5 ppm and β/γ -protons at δ 1.4 - 4.0 ppm of peptide bonds (Figure 2). Additionally we can observe multiple sets of A_2B_2 coupling pattern protons of benzene rings at δ 6.6 - 7.1 ppm (each d, $J \sim 8.0$ Hz), methylene protons of long aliphatic chains at δ 1.0 - 1.3 ppm and terminal methyl protons at δ 0.7 - 1.0 ppm. These patterns of signals are consistent with the presence, almost exclusive, of lipopeptides, compounds made of amino acids and a fatty acid chain [14] [23]-[25].

A LC-ESI-MS spectral analysis of the cell free supernatant showed a cluster containing molecules that were observed at m/z 1031, 1045 1463 and 1477 (Figure 3). These peaks differ by 14 Da suggesting a series of homologous molecules with different lengths of fatty acid chain. In the literature bacterial lipopeptides such as iturin A, surfactin and fengycin exhibited the same peak profile that the obtained with *Bacillus* sp. CCMI1053. Kim *et al.* (2010) described that the strains of *Bacillus subtilis* CMB32 produced lipopeptides with molecular masses estimated by 1080, 1486 and 1044 Da, corresponding to the antifungal lipopeptides iturin A, fengycin and surfactin A, respectively [26]. Caldeira *et al.* (2011) reported that *Bacillus amyloliquefaciens* CCMI1051 exhibit high levels of antagonistic properties against filamentous fungi duo to the production of compounds of masses between 1000 and 1100 Da, comparable to that of iturin and surfactin compounds between 1436 and 1478 Da, compatibles with fengycin [17].

Thus, the peaks with m/z 1031, 1045, 1463 and 1477 could be assigned to lipopeptides. The mass spectra obtained in ESI mode shows a cluster containing molecules with a difference of 14 Da, corresponding to the loss of CH_2 in the lipidic chain [9] (m/z 1031, 1045 and 1463, 1477) (Figures 3(a)-(d)). Several isoforms exist for each li-

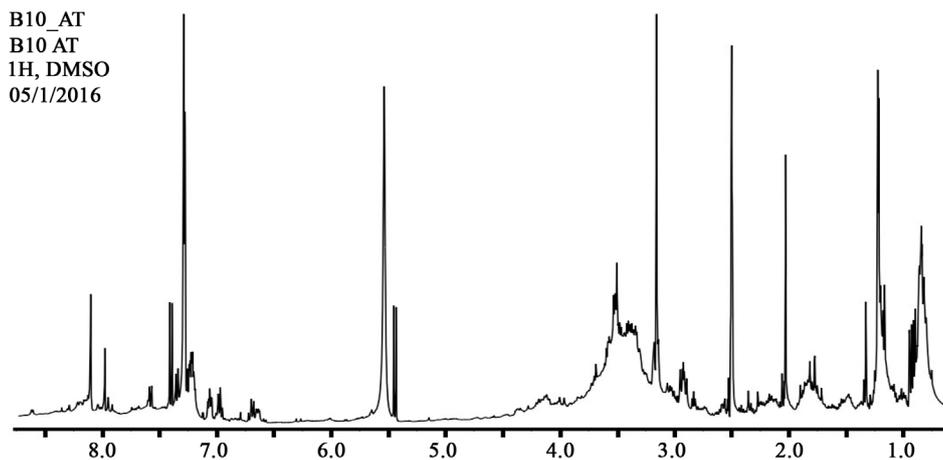


Figure 2. ¹H-NMR spectra of the bioactive compounds produce by *Bacillus* sp. CCM1053.

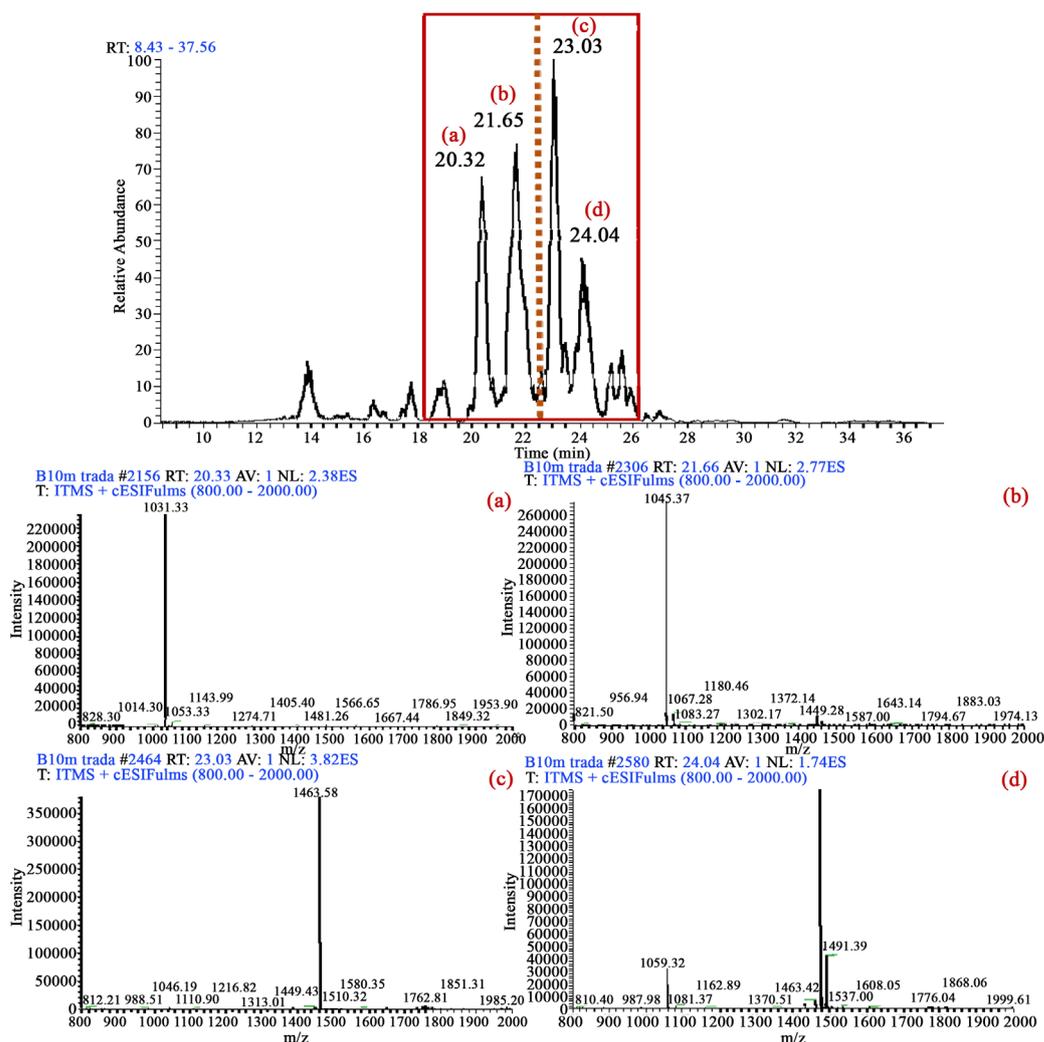


Figure 3. Total ion chromatogram of the cell free supernatant concentrated by column chromatography of *Bacillus* sp. CCM1053 and mass spectra corresponding to the peaks (a) m/z 1031.37; (b) m/z 1045.37; (c) m/z 1463.53 and (d) m/z 1477.55.

peptide due to diverse producer strains and different nutritional conditions. These environments affect the substitution of amino acids in the peptidic ring and the length of lipidic chain [27].

4. Conclusion

In this work, a combined use of NMR and LC-ESI-MS allows a rapid identification of lipopeptides produced by *Bacillus* sp. CCMI1053. The proposed methodology allowed to access bioactive metabolites produced without need of the laborious total previous isolation and could be used as a viable alternative to be employed for screening and production of new green biocides.

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