Novel Biocides for Cultural Heritage

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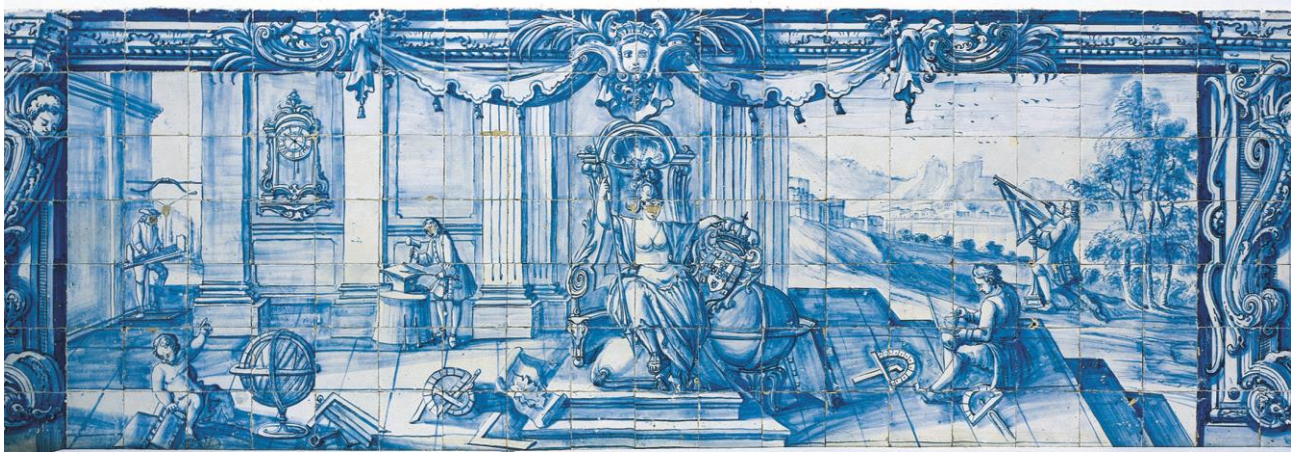

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"The very essence of instinct is that it's followed independently of reason."

Charles Darwin

Aos meus pais e Pedro
To my parents and Pedro

"A ciência descreve as coisas como são; a arte, como

são sentidas, como se sente que são."

Fernando Pessoa

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Abstract

Many microorganisms, influenced by environmental conditions, are the main responsible for biological contamination in built heritage. Biocides based on chemical toxic compounds have been the most often used to mitigate this problem. Thus, it is of vital importance to develop proper remediation actions based on environmentally innocuous alternatives. Bacteria of the genera *Bacillus* are emerging as an optimistic alternative due to their capacity to produce secondary metabolites with antagonistic activities against many fungal pathogens.

This work aimed to develop ground-breaking research in the area of cultural and built heritage rehabilitation, by the development of natural and green safe biocides for biodegradation/biodeterioration treatment of Cultural Heritage.

A complementary methodology, including antifungal tests and molecular approaches was used, in combination with microscopic and analytical techniques to detect, characterise and study the efficiency of the biological active compounds produced by *Bacillus* sp. strains.

Flow cytometry allowed a comprehensive study of the physiological mechanism behind the bioactive compounds production in order to understand and improve the strategic approaches for process optimisation and scale up production. Moreover, according to the results of the toxicological tests, these compounds have proven to be a real environmental safe and innocuous alternative to the chemical biocides commonly used during the conservative interventions. Thus, they have shown a great potential for their future application in cultural and built heritage rehabilitation.

Keywords

Biodegradation/Biodeterioration, *Bacillus* sp., Bioactive compounds, Lipopeptides, Iturin, Antifungal activity, Sporulation, Green Biocides, Cultural Heritage rehabilitation

Novos Biocidas para o Património Cultural

Resumo

Vários microrganismos influenciados pelas condições ambientais são os principais responsáveis pela contaminação biológica do património cultural edificado. Na tentativa de mitigação destes agentes, compostos geralmente tóxicos têm sido os mais utilizados. Assim, é de enorme importância desenvolver ações de remediação dirigidas aos agentes efetivamente biodeteriogénicos, baseados em alternativas inócuas para o meio ambiente. As bactérias do género *Bacillus* surgem, como uma viável alternativa devido à capacidade de produzir metabolitos secundários com atividade antagonista, contra diversos fungos.

Este trabalho teve como objetivo desenvolver uma investigação inovadora que possa vir a ser útil na área de reabilitação do património cultural edificado, através da produção de novos biocidas naturais e mais ecológicos.

Utilizou-se uma abordagem metodológica, que incluiu testes antifúngicos e abordagens moleculares, combinadas com técnicas microscópicas e analíticas, de forma a detetar, caracterizar e estudar a eficiência de compostos biologicamente ativos produzidos por estripes de *Bacillus* sp.. Foram ainda utilizados os mecanismos fisiológicos por detrás da produção destes compostos, de forma a perceber e melhorar as abordagens estratégicas no processo de otimização da produção. Em testes toxicológicos, compostos produzidos por estirpes de *Bacillus* sp. selecionados, provaram ser uma alternativa ecológica aos biocidas químicos, comumente utilizados em intervenções de conservação. Desta forma, estes demonstram um elevado potencial para futura utilização na reabilitação do património cultural edificado.

Palavras-chave

Biodegradação/Biodeterioração, *Bacillus* sp., Compostos bioativos, Lipopéptidos, Iturina, Atividade antifúngica, Esporulação, Biocidas ecológicos, Reabilitação do Património Cultural

List of Publications

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Table of Contents

Acknowledgments.....V

Agradecimentos.....IX

Abstract.....XIII

Resumo.....XV

List of Publications.....XVII

Table of Contents.....XIX

List of Figures.....XXIV

List of Tables.....XXIX

Abbreviations.....XXXI

Units.....XXXIII

Aims and Methodology.....1

Chapter I. Introduction.....3

1.1. Cultural Heritage biodeterioration/biodegradation.....5

1.1.1. Mitigation approaches.....9

1.1.2. Biocides treatment.....13

1.2. Biosurfactants compounds – a natural solution.....17

1.2.1. Lipopeptides production.....19

1.2.2. *Bacillus* sporulation.....26

1.2.3. Lipopeptides biosynthesis.....33

1.2.4. Lipopeptide detection and characterisation.....37

Chapter II. Microorganisms, producers of Lipopeptides 43

1. Overview 45

2. Introduction 46

3. Materials and Methods 48

3.1. Bacterial strains and culture media 48

3.2. Bioactive compounds production 49

3.3. Antifungal activity assessment 49

3.4. Liquid cultures growth conditions 49

3.5. Bacterial DNA extraction 50

3.6. 16S ribosomal DNA sequence analysis 51

3.7. Amplification of *Bacillus* lipopeptide genes 52

3.8. Real-time PCR analysis 53

3.9. LC-ESI-MS analysis 54

4. Results and Discussion 55

4.1. Selection of bacterial strains with antifungal potential 55

4.1.1. Screening antifungal activity 55

4.1.2. Amplification of *Bacillus* lipopeptide genes 57

4.2. Quantification of iturin genetic expression 59

4.3. Identification and characterisation of bacterial strains with antifungal potential 61

4.4. Molecular phylogeny analysis 67

4.5. Physiological characterisation 69

5. Conclusions 72

Chapter III . Bioactive compounds detection: Antifungal potential in cultural heritage context 73

1. Overview 75
2. Introduction 76
3. Materials and Methods 78
   3.1. Microorganisms maintenance 78
   3.2. Growth conditions and bioactive compounds production 79
   3.3. Antifungal activity of bioactive compounds 79
      3.3.1. Antifungal paper disks diffusion assay 79
      3.3.2. Interaction between novel biocides and heritage biodeteriogenic fungi 80
      3.3.3. Statistical analyses 80
   3.2. Column chromatography 81
   3.3. LC–ESI-MS analysis 81
   3.4. Nuclear magnetic resonance (NMR) 82
   3.5. Bioautographic detection 82
4. Results and Discussion 83
   4.1. Antifungal activity of bioactive compounds 83
   4.2. Characterisation of the antifungal compounds 88
5. Conclusions 97

Chapter IV. Physiology of *Bacillus* with antifungal proprieties: lipopeptides production 99

1. Overview 101
2. Introduction 102
3. Materials and Methods 105
   3.1. Microorganism and culture media 105
   3.2. *Bacillus* growth monitoring 105
   3.3. Spores formation assessment 105
   3.4. Physiological monitorisation 106

3.5. Monitorisation of LP production 107
3.6. Spores germination of *Bacillus* sp. CCLBH 1053 108
4. Results and Discussion 110
4.1. Cell growth dynamics of *Bacillus* sp. CCLBH 1053 110
4.2. *Bacillus* sporulation assessment 111
4.3. Antifungal compounds production 113
4.4. Cells population analysis by flow cytometry 116
4.3.1. NB culture medium analysis 117
4.3.2. LAPM culture medium analysis 122
4.5. Spore germination study 126
5. Conclusions 132

Chapter V. Toxicological assessment and antifungal efficiency of the bioactive compounds 135

1. Overview 137
2. Introduction 138
3. Materials and Methods 141
3.1. Microorganisms and Bioactive compounds production 141
3.2. Acute toxicity assessment 141
3.2.1. Toxicity in *Artemia salina* 141
3.2.2. Animals 142
3.2.3. Acute toxicity in *Swiss* mice 143
3.2.4. Statistical analyses 144
3.3. Simulation assays with mortar samples 145
3.3.1. *In vitro* assay using mortars slabs 145
3.3.2. Real mortars under the presence of the new biocides 146
4. Results and Discussion 147
4.1. Toxicological evaluation 148
4.1.1. Toxicity in *Artemia salina* 149

4.1.2. Acute toxicity in *Swiss* mice 151

4.2. New compounds effectiveness in cultural heritage context 152

4.2.1. Simulation *in vitro* assays using mortars slabs 152

4.2.2. Monitorisation of artificially inoculated fragments of mortars from mural painting 155

5. Conclusion 158

Chapter VI. Concluding Remarks 159

References 165

Annexes 189

Annexe A. Culture media composition 191

Annexe B. Solutions composition 192

Annexe C. *Bacillus* 16S ribosomal DNA amplification and agarose gel electrophoresis 193

Annexe D. Determination of DNA extracted from the bacteria cells 194

Annexe E. Multiple alignment of the rDNA 16s sequence of the *Bacillus* sp. strains and the most similar *Bacillus* sequence found for each one in the Gene Bank sequence database 195

Annexe F. Toxicity assay 199

Annexe G. Statistical analysis 200

List of Figures

Chapter I.

Figure I-1: Different artworks with signals of deterioration caused fungal communities. A - Renaissance Frescoes from Santo Aleixo Church, Montemor-o-Novo, Portugal, B- Mural paintings from *Casa Pintadas*, Évora, Portugal, C – Mural paintings from *Santa Clara* Church (Sabugueiro, Arraiolos, Portugal), D- Gilded woodcarving from altars of the *Espírito Santo Church*, Évora, Portugal. 7

Figure I-2: Work of preventive cultural heritage conservation. A- Application of mechanical methods in a rock wall with prehistoric paintings at the Serra da Capivara, National Park, Brasil. B- Conservator-restorer cleaning mold with chemical toxic biocide. 10

Figure I-3: Structure of a surfactin member synthesised by *Bacillus* species.. 22

Figure I-4: Structure of an iturin member synthesised by *Bacillus* species..... 24

Figure I-5: Structure of fengycin member synthesised by *Bacillus* species. 25

Figure I-6: The key stages of the sporulation cycle of *Bacillus subtilis*.. 27

Figure I-7: Cell division during *Bacillus subtillis* growth. 28

Figure I-8: Chromosome partitioning and *Bacillus subtillis* asymmetric division. 29

Figure I-9: *Bacillus* cell-specific activation of σ^F. 30

Figure I-10: Cross-section of a spore of *Bacillus subtilis*, showing the cortex and coat layers surrounding the core (dark central area). The spore is 1.2 microns across, about 100 times smaller than the width of a human hair. 32

Figure I-11: Organisational structure of peptide synthetases located outside of the ribosome. 33

Figure I-12: Structural organisation of the genes encoding surfactin and iturin biosurfactant synthetases family.. 36

Figure I-13: Structural organisation of the genes encoding fengycin synthetases family. 37

Figure I-14: Multianalytical approaches to *Bacillus* lipopeptides detection. 38

Chapter II.

Figure II-1: Schematic diagram of mycosubtilin, bacillomycin D and iturin A operon from Moyne *et al*., 2004. 47

Figure II-2: Agarose gel electrophoresis of DNA isolated from different selected strains. A- CCLBH 1051; B- CCLBH 1052; C- CCLBH 1053; D- CCLBH 1054; Lane

1- 100 bp Ladder (Nzytech Ladder VII); Lane 2- PCR product of bacterial amplification with ituA primer; Lane 3- PCR product of bacterial amplification with ituB primer; Lane 4- PCR product of bacterial amplification with ituC primer; Lane 5- PCR product of bacterial amplification with ituD primer..... 58

Figure II-3: Iturinic ORF's amplified DNA copies in the genome of the selected bacteria strain determined with real time PCR. (■ itu A; ▨ itu B; ■ ituC; 〓 ituD). 59

Figure II-4: Phylogenetic relationships of *Bacillus* species based on nucleotide sequences of the 16S rDNA. The tree was generated by the neighbor-joining method (MEGA 4.0 software)..... 68

Figure II-5: Time course profiles of *Bacillus* sp.. *Bacillus* CCLBH sp. 1051 (–●–); *Bacillus* sp. CCLBH 1052 (–o–); *Bacillus* sp. CCLBH 1053 (–▼–); *Bacillus* CCLBH sp. 1054 (–Δ–). 69

Figure II-6: Total ion chromatogram of the supernatant liquid culture of *Bacillus* sp. CCLBH 1051 and mass spectra corresponding to the peaks (a) m/z 1031.36 and (b) m/z 1045.37. 71

Chapter III.

Figure III-1. Antifungal activity of biocides against *Cladosporium* sp. using the paper disk diffusion assay. A- Control. B- In the presence of 10 µL of *Bacillus* sp. CCLBH 1053 cell-free culture Broth. 85

Figure III-2: Percentage of inhibition growth resulted from interaction assays between biodeteriogenic fungi and *Bacillus* sp. cell-free culture broth. (■ *Penicillium* sp.1, ■ *Alternaria* sp., ■ *Mucor* sp., ■ *Fusarium oxysporum,* ■ *Cladosporium* sp., ■ *Penicillium* sp. 2, ■ *Aspergillus niger).* Different letters (a-m) following the values indicate significant differences ($p<0.05$). Values of each determination represents means ± SD (n=3)..... 87

Figure III-3: Total ion chromatogram of the supernatant liquid culture of *Bacillus* sp. CCLBH 1051 and mass spectra obtained for the retention times (a) m/z 1031.35 and (b) m/z 1045.40..... 90

Figure III-4: Total ion chromatogram of the supernatant liquid culture of *Bacillus* sp. CCLBH 1052 and mass spectra obtained for the retention times (a) m/z 1031.27, (b) m/z 1045.31 and (c) m/z 1464.07. 91

Figure III-5: Total ion chromatogram of the supernatant liquid culture of *Bacillus* sp. CCLBH 1053 and mass spectra obtained for the retention times (a) m/z 1031.37, (b) m/z 1045.37 and (c) m/z 1463.53. 92

Figure III-6: ^{1}H-NMR spectra of the bioactive compounds produced by *Bacillus* sp. CCLBH 1053. 94

Figure III-7: Autobiographical results. Chromatographic scheme profile. A- The *Bacillus* sp. CCLBH 1053 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 imagem); C- Bioautograms with TLC layers applied separately from *Bacillus* CCLBH 1053. 95

Figure III-8: FTIR-ATR analyses of the biologically active compounds produced by *Bacillus* CCLBH 1053 (I- Rf = 0.429) on the bioautographic plate. (—— *Bacillus* sp. CCLBH 1053; —— Silica) 96

Chapter IV.

Figure IV-1: Methodological scheme of inducing germination assay..... 109

Figure IV-2: Time course profiles of *Bacillus* sp. CCLBH 1053. NB medium (●); LAPM medium (●). All data was determined in triplicate. 111

Figure IV-3: Concentration of particles in *Bacillus* sp. CCLBH 1053 in NB (A) and LAPM (B) liquid culture. (●) [Cell] (cell/mL); (■) [Spo] (CFU/mL); (◆) [Spo]/[Cell] (CFU/cell). The marked area evidence the strongest variations observed..... 112

Figure IV-4: Total ion chromatogram of the cell free supernatant of *Bacillus* sp. CCLBH 1053 and mass spectra corresponding to the peaks (a) m/z 1031.33, (b) m/z 1045.37 and (c) m/z 1463.56, after 48 h of culture. 113

Figure IV-5: Lipopeptide production of *Bacillus* sp. CCLBH 1053 culture along culture time for NB culture medium (A) and LAPM medium (B) (◆ m/z 1463; ● m/z 1031; ▲ m/z 1045). 115

Figure IV-6: Variation of cell population distribution, of *Bacillus* sp. CCLBH 1053 growth in NB, along time assessed by staining with 7-AAD and Annexin V followed and flow cytometry analysis. A- (●) Late apoptotic cells; (■) Early apoptotic cells; (◆) Total apoptotic cells. B- (■) Live cells; (●) Dead cells. The marked area evidence the strongest variations observed. 118

Figure IV-7: Time course of the two sub-populations of *Bacillus* sp. CCLBH 1053 growing in NB medium. A, B- Dot-plot cytometry and histograms at 1 and 96 hours of incubation, respectively. (a)-(e)- No stained cells; (f)-(j) - Cells stained with a mixture of Annexin V and 7-AAD dyes. (a), (c), (f), (h) - Cells sensed with Annexin V detector; (b), (d), (g), (i) - Cells sensed with 7-AAD detector. (e), (j) - Flow cytometer forward scatter (FSC) for cell size analysis..... 120

Figure IV-7 (continued): Time course of the two sub-populations of *Bacillus* sp. CCLBH 1053 growing in NB medium. C, D- Dot-plot cytometry and histograms at 168 and 720 hours of incubation, respectively. (a)-(e)- No stained cells; (f)-(j) - Cells stained with a mixture of Annexin V and 7-AAD dyes. (a), (c), (f), (h) - Cells sensed with Annexin V detector; (b), (d), (g), (i) - Cells sensed with 7-AAD detector. (e), (j) - Flow cytometer forward scatter (FSC) for cell size analysis. 121

Figure IV-8: Variation of cell population distribution, of *Bacillus* sp. CCLBH 1053 growth in LAPM medium, along time assessed by staining with 7-AAD and Annexin V followed and flow cytometry analysis. A- (●) Late apoptotic cells; (■) Early apoptotic cells; (◆) Total apoptotic cells. B- (■) Live cells; (●) Dead cells..... 123

Figure IV-9: Time course of the two sub-populations of *Bacillus* sp. CCLBH 1053 growing in LAPM medium. A, B- Dot-plot cytometry and histograms at 1 and 120 hours of incubation, respectively. (a)-(e)- No stained cells; (f)-(j) - Cells stained with a mixture of Annexin V and 7-AAD dyes. (a), (c), (f), (h) - Cells sensed with Annexin V detector; (b), (d), (g), (i) - Cells sensed with 7-AAD detector. (e), (j) - Flow cytometer forward scatter (FSC) for cell size analysis. 124

Figure IV-9 (continued): Time course of the two sub-populations of *Bacillus* sp. CCLBH 1053 growing in LAPM medium. C, D- Dot-plot cytometry and histograms at 168h and 720 hours of incubation, respectively. (a)-(e)- No stained cells; (f)-(j) - Cells stained with a mixture of Annexin V and 7-AAD dyes. (a), (c), (f), (h) - Cells sensed with Annexin V detector; (b), (d), (g), (i) - Cells sensed with 7-AAD detector. (e), (j) - Flow cytometer forward scatter (FSC) for cell size analysis. 125

Figure IV-10: Time course profile of *Bacillus* cells (A) and spores formation (B), before and after the peptone supplementation and heat activation assays. (■) Normal growth in NB culture medium; (●) NBPSHA assay; (◆) NBPS assay. 127

Figure IV-11: Lipopeptide production increment for NBPS assay and NBPSHA assay. The relative percentage of increase was calculated considered as 100% the area of peak corresponding to 0 h of incubation time after treatment. (—●—) NBPSHA; (—●—) NBPS assay. 131

Chapter V.

Figure V-1: Schematic representation of the acute toxicity assay on *Swiss* mice. 143

Figure V-2: *Swiss* mice toxicity assay. A- Pineal reflexes tests, B- Motor activity teste, C- Traction tests and D- Behaviour observation in toxicological evaluation assay. 144

Figure V-3: Methodological scheme of simulation *in vitro* assays using mortars slabs and real samples under the presence of bioactive compounds. 147

Figure V-4: % of *Artemia salina* mortality for the bioactive compounds produced and for the commercial biocides at a concentration of 1000 µg/mL in each well. Different letters (a-b) following the values indicate significant differences ($p<0.05$). Values of each determination represents means ± SD (n=3). 149

Figure V-5: Slabs mortars, SEM micrographs of the MS1 microfragments and EDS analysis. A-E: Control slabs inoculated with *Cladosporium* sp.; F-H: MS with CB 3 (bioactive compounds produced by *Bacillus* sp. CCLBH 1053). 153

Figure V-6: Bi-dimensional projection of International Commission on Illumination a* b* chromatic coordinates of the 12 measurements that were performed on the real mortars of the Santo Aleixo's mural painting. ▲ Control; ● RM1; ■ RM2. 157

Annexes.

Figure C-1: Agarose gel electrophoresis of 16S ribosomal DNA isolated from different *Bacillus* sp. strains. Legend: Lane 1- 100 bp Ladder (Nzytech Ladder VII); Lane 2- PCR product of 16S rDNA amplification of *Bacillus* sp. CCLBH 1051; Lane 3- PCR product of 16S rDNA amplification of *Bacillus* sp. CCLBH 1052; Lane 4- PCR product of 16S rDNA amplification of *Bacillus* sp. CCLBH 1053; Lane 5- PCR product of 16S rDNA amplification of *Bacillus* sp. CCLBH 1054. 193

List of Tables

Chapter I.

Table I-1: Chemical biocides used in artworks treatment. 14
Table I-2: Structural variants diversity within the surfatin lipopeptide family synthesised by *Bacillus* species. 21
Table I-3: Structural variants diversity within the iturin lipopeptide family synthesised by *Bacillus* species. 23
Table I-4: Structural variants diversity within the fengycin lipopeptide family synthesised by *Bacillus* species. 25

Chapter II.

Table II-1: Oligonucleotide primers used to detect genetic markers for biological activity in bacteria with antifungal potential. 52
Table II-2: Antifungal activity assay with 21 bacteria strains isolated against biodeteriogenic fungi. 56
Table II-3: Macroscopic and microscopic appearance of the selected microorganisms in analysis. 62
Table II-4: Homology search from the Ribosomal database (RDP) of the CCLBH 1051 16S rDNA. 63
Table II-5: Homology search from the Ribosomal database (RDP) of the CCLBH 1052 16S rDNA. 64
Table II-6: Homology search from the Ribosomal database (RDP) of the CCLBH 1053 16S rDNA. 64
Table II-7: Homology search from the Ribosomal database (RDP) of the CCLBH 1054 16s rDNA. 64
Table II-8: Specific growth rate and generation time values for the *Bacillus* sp. strains selected. 70

Chapter III.

Table III-1. Antifungal activity of bacterial bioactive compounds and commercial antifungal drugs against the biodeteriogenic fungi, isolated from deteriorated mural paintings. 84

Chapter IV.

Table IV-1: Flow cytometer forward scatter (FSC), fluorescence histograms of *Bacillus* sp. CCLBH 1053 culture in NB and of two different sporulating assays in the same medium with Peptone Supplementation (NBPS) only, or followed by heat shock (NBPSHA) in different time of incubation. 129

Chapter V.

Table V-1: LC_{50} parameter of biocides tested. 150
Table V-2: Schematic representation of the lab experiments conducted on real mortars samples. RM1- Mortar impregnated with extracted compounds from *Bacillus* sp. CCLBH 1053; RM2- Mortar impregnated with CB 3. 156

Annexes.

Table A-1: Composition of the culture media used for microbiological growth. 191
Table D-1: DNA concentration extracted from of the bacteria strains in analysis. . 194
Table F-1: Composition of the saline medium used to *Artemia salina* toxicity assay. 199
Table G-1: Analysis of variance (ANOVA) of interaction assay against biodeteriogenic fungi..... 200
Table G-2: Average multiple comparison of interaction liquid assay against biodeteriogenic fungi, by Tukey HSD test. 201
Table G-3: Analysis of variance (ANOVA) of the *Artemia salina* toxicity assay for bioactive compounds produced and commercial biocides..... 202
Table G-4: Average multiple comparison of the *Artemia salina* toxicity assay for bioactive compounds produced and commercial biocides..... 203
Table G-5: Analysis of variance (ANOVA) of the bi-dimensional projection of CIElab chromatic coordinates of the real mortars from mural painting. 204
Table G-6: Average multiple comparison of the bi-dimensional projection of CIEla*b* chromatic coordinates, by Tukey HSD test. 204

Abbreviations

μ	Specific growth rate
7-ADD	7-aminoactinomycin D
A	Adenine nucleotide
A	Adenylation domain
Ala	Alanine
ANOVA	Analysis of variance
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine triphosphate
BDP	Biocidal Product Directive
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
C	Condensation domain
C	Cytosine nucleotide
CB	Compounds Bioactive
CF	Cycle fluorescence
cLPP	Cyclic Lipopeptide
CRB	Cook Rose Bengal
Ct	Threshold cycle
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DPA	Dipicolinic acid
e	*Neper* number
E	Epimerization
EC	European Comission
EDS	Energy Dispersive X-ray Spectroscopy
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCM	Flow cytometry
FELASA	Federation of European Laboratory Animal Science Associations
FSC	Flow cytometer forward scatter forward scatter
FTIR-ATR	Fourier Transform Infrared spectroscopy-Attenuated Total Reflection
g	Time of generation
G	Guanine nucleotide
GC	Gas chromatography
Glu	Glutamic acid
HPLC	High performance liquid chromatography
Ileu	Isoleucine
IR	Infrared spectroscopy
LAPM	Lipopeptide Antibiotic Production medium
LC_{50}	Lethal concentration 50%
LC–ESI-MS	Liquid chromatography coupled with mass spectrometry/ Electrospray ionisation

LD_{50}	Lethal dose 50%
Leu	Leucine
LPP	Lipopeptide
MALDI-TOF	Matrix-Assisted Laser Desorption Ionisation/Time-Of-Flight
MEA	Malt Extract Agar
MS	Mass spectroscopy
MS	Mortar Slabs
NA	Nutrient Agar
NaOH	Sodium hydroxide
NB	Nutrient Broth
NBPS	Nutrient Broth Peptone Supplementation assay
NBPSHA	Nutrient Broth Peptone Supplementation and Heat-Activation assay
NCBI	National Center for Biotechnology Information
NH_4	Ammonium
NMR	Nuclear magnetic resonance
NRPS	Nonribosomal peptide synthetase
OECD	Organisation for Economic Co-operation and Development
ORF	Open Reading Frame
Orn	Ornithine
p	p-value
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PI	Propidium iodide
PKS	Polyketide synthases
Pro	Proline
PS	Phosphatidylserine
rDNA	Ribosomal Deoxyribonucleic Acid
RDP	Ribosomal Database Project
Rf	Retention Factor
RFU	Relative fluorescence units
RM	Real mortar
RNA	Ribonucleic Acid
rRNA	Ribossomal Ribonucleic Acid
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscopy
SEM-EDS	Scanning Electron Microscopy coupled with Energy Dispersive X-ray Spectroscopy
T	Thymine nucleotide
T	Thiolation domain
Thr	Threonine
TLC	Thin-layer chromatography
Tyr	Tyrosine
UV	Ultraviolet light
Val	Valine

Units

%	Percentage
A	Ampere
Bp	Base pairs
CFU	Colony-forming unit
cm	Centimetre
Da	Dalton
g	Gram
h	Hour
Hz	Hertz
m/z	Mass to charge ratio
min	Minute
nm	Nanometer
ºC	Celsius degree
ppm	Part per million
rpm	Rotation per minute
s	Second
V	Volt
v	Volume
w	Weight

Aims and Methodology

Biodeterioration is an undesirable process, triggered by living organisms, which can affect cultural and built heritage and economically important materials. The importance of carrying out proper remediation actions for microbiologically contaminated historic materials is of vital importance. The growth control of microflora in cultural and built heritage is usually done by treatments using chemical compounds that have high toxicity to humans. *Bacillus* species can be worth for these treatments because they produce a great diversity of secondary metabolites known to possess antagonistic activity against many fungal pathogens.

Thus, the main goal of this project is to obtain and study new bioactive molecules produced by different strains of *Bacillus* sp., to prove their remediation potential and to establish preventive approaches in heritage and construction context. For this reason, several methodological approaches, including microorganism and DNA manipulations, microscopic, spectrometric, spectroscopic and cytometric techniques, in combination with simulation assays, were developed in order to establish effective tools for the study of active compounds obtained from natural sources, against biodeteriogenic agents of artistic heritage.

The methodology defined for this work intended:

- To select microorganism producers of compounds with antifungal potential;
- To identify and characterise the bacterial strains with higher activity against biodeteriogenic fungi isolated from biodegraded cultural heritage artefacts;
- To determine kinetic features for the selected bacterial strains;
- To develop a combined methodology for quick identification of bioactive compounds-producing strains;

- To evaluate the antifungal potential against heritage biodeteriogenic fungi using different antifungal activity approaches;
- To define methodological basis for bioactive compounds detection directed to heritage biodeteriogenic fungi;
- To characterise the bioactive metabolites using spectroscopic analyses, including FTIR-ATR, ^{1}H- NMR and LC–ESI-MS analysis;
- To monitor the physiology of the *Bacillus* sp. cells by multi-parameter flow cytometry and the physiological response to nutrient starvation and re-supplementation;
- To characterise, interpret and understand the compounds production and the relation with cell viability and sporulation;
- To evaluate the toxicological properties of the new bioactive compounds produced using two different biological models: brine shrimp (*Artemia salina*) and Swiss mice (*Mus Muculus*);
- To study the real life efficiency and influence of these new compounds in the growth of biodeteriogenic fungi, using simulation assays.

CHAPTER I

Introduction

1.1. Cultural heritage biodeterioration/biodegradation

The preservation of historic monuments and buildings, which represent the cultural heritage of a country, constitutes a high societal priority in order to give the opportunity for the future generations to witness their ancestors achievements (Steinbauer *et al.*, 2013).

The problems caused by lack of proper preservation of historical built heritage often only come to attention when a tragedy, such as a fire or collapse, occurs. Unfortunately silence threats lurking permanently in our cultural heritage, far from the eyes of the great majority of the people.

Nowadays, science and technology interact with art in several ways. In fact, the combination of biotechnological an analytical approaches can play an important role in protecting and preserving cultural heritage for future generations (Fernandes, 2006).

Environmental factors (humidity, temperature, light, CO_2 concentration, atmospheric pressure and pH), geological conditions of the ground, chemical composition (organic and inorganic nutrient sources), quality and ageing of the materials, internal mechanical stress and biological agents constituted the main parameters that influence artworks decay (Nugari *et al.*, 2009; Pangallo *et al.*, 2009; Capodicasa *et al.*, 2010; Gaylarde *et al.*, 2011; Tran *et al.*, 2012; Rosado *et al.*, 2014).

Whereas several biotic and abiotic factors can induce degradation/deterioration, the action of the biotic factors was neglected for a long time, and the abiotic factors were the only ones taken into account (Rojas *et al.*, 2009).

Biodegradation/biodeterioration can be defined as “any undesirable change in a material brought about by the vital activities of organisms” (Sterflinger and Piñar, 2013). This phenomenon is an undesirable process, triggered by living organisms, which can affect cultural and built heritage and economically important materials (Sterflinger, 2010; López-Miras *et al.*, 2013; Sterflinger and Piñar, 2013)

Microorganisms, including bacteria, fungi, algae and lichens as well as insect pests, influenced by environmental conditions, are the main biodeteriogenic agents responsible for aesthetical and structural damage of cultural heritage (Rosado *et al.*, 2013a), causing problems in its conservation. This holds true for all types of historic artefacts and even for art made of modern materials (e.g., polymers) from public museums and from private art collections (Sterflinger and Piñar, 2013).

In the specific case of mural paintings, the development of microorganisms may cause discolouration of pigments and mortars, formation of stains and biofilms, salt efflorescence appearance, exfoliation of paint layers, formation of paint blisters, cracking and disintegration of paint layers, and degradation of binders that results in detachment of the paint layer (Guiamet *et al.*, 2011; Borrego *et al.*, 2012; López-Miras *et al.*, 2013; Sterflinger and Piñar, 2013).

Despite the involvement of microorganisms in the deterioration process is well known, the specific role of the different groups and species that integrate the microbial communities is not yet well understood. A wide diversity of microorganisms are involved in artworks deterioration. Among them, fungi are particularly dangerous because their hyphae may have high level of proliferation in the materials and their spores, in a dormant state, are commonly present and available for germination but also because fungal derived carboxylic acids (e.g., oxalic, citric, succinic, formic, malic, acetic, fumaric, glyoxylic, gluconic, and tartaric acids) can induce chemical attack. Fungi of the genera *Penicillium*, *Cladosporium, Alternaria*, *Curvularia*, *Dreschlera*, *Chaetomium*, *Fusarium*, *Trichoderma*, *Gliomastix*, *Aureobasidium*, are the most abundant in degraded mural paintings (Figure I-1, A-C).

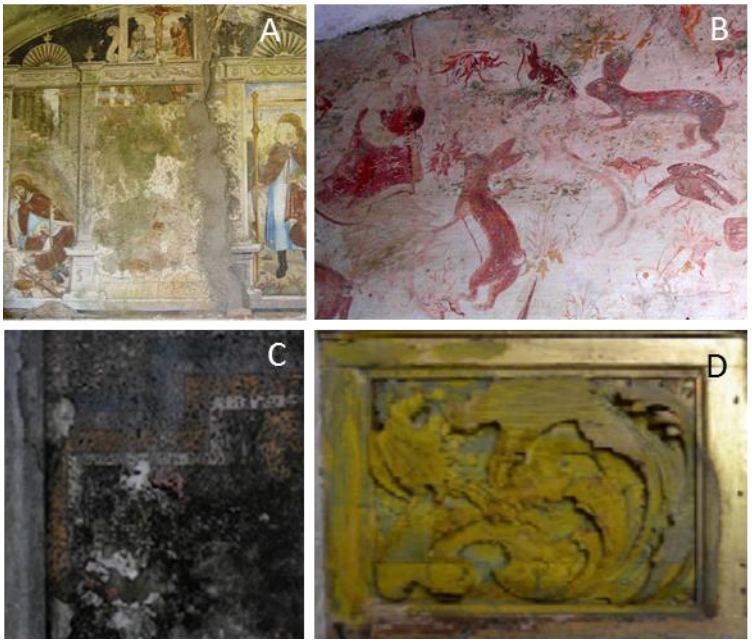


Figure I-1: Different artworks with signals of deterioration caused fungal communities. A - Renaissance Frescoes from *Santo Aleixo* Church, Montemor-o-Novo, Portugal, B- Mural paintings from *Casa Pintadas*, Évora, Portugal, C – Mural paintings from *Santa Clara* Church (Sabugueiro, Arraiolos, Portugal), D- Gilded woodcarving from altars of the *Espírito Santo Church*, Évora, Portugal. Adapted from (Rosado et al., 2013b; Rosado et al., 2014; Rosado et al., 2015a; Rosado et al., 2015b).

Stone and other building materials, such as concrete, mortar, slurries, paint coatings, glass and metals used in architecture are object of overall deterioration phenomena in which different sorts of microorganisms are involved (Scheerer *et al.*, 2009). On building stone exposed to open air, fungi may also be the most important biodeteriorative organisms because of their extremely erosive potential (Scheerer *et al.*, 2009; Sterflinger and Piñar, 2013).

Depending on the physical properties of the material, fungi may penetrate or not inside the stone. There are two major morphological and ecological groups of stone-inhabiting and stone-dwelling fungi. These have adapted to different environmental conditions. In moderate or wet weather, the fungal communities on rock are dominated by hyphomycetes that form mycelia (hyphal networks) in the porous space of the stones (Sterflinger, 2010). Since the first step for fungal colonisation is the settlement of spores

from the air, the species diversity of stone fungi is rather similar to the diversity of common airborne spores. *Alternaria*, *Cladosporium*, *Epicoccum*, *Aureobasidium* and *Phoma* are the most important fungal strains (Sterflinger, 2000). However, due to their thick walls, fungi can resist to chemical attack and, therefore, resist biocides and other anti-microbial treatments.

Cyanobacteria, algae and lichens contribute to the weathering of stone in humid as well as in semi-arid and arid environments, producing a characteristic phenomenon consisting of large green-black stains (Cutler *et al.*, 2013).

Additionally, the role of chemoheterotrophic bacteria in the weathering of rock probably depends largely on the environmental conditions. While bacteria might growth in humid environments and form biofilms within the porous space of building stone, the occurrence of chemoheterotrophic bacteria might be more limited (Lamprinou *et al.*, 2013).

The excretion of inorganic acids (e.g. nitric and sulfuric acids) on rock surfaces, by chemolithotrophic bacteria, that use carbon dioxide as carbon source, produce energy through the oxidation of inorganic compounds (electron donors) such as ammonium (NH_4), nitrogen dioxide and hydrogen sulphide (Sáiz-Jiménez and Laiz, 2000). Another way consist in the excretion of organic acids in surfaces of the monuments by fungi and bacteria chemoorganotrophic - those obtain energy by oxidising organic molecules or other living beings. Microorganisms use these organic substrates to increase their population and activity - and consequently, the biodeterioration process (Gaylarde *et al.*, 2011; Cutler *et al.*, 2013).

Moreover, a well-known phenomenon often observed on buildings and wall paintings is the formation of salt efflorescence on surfaces (Sáiz-Jiménez and Laiz, 2000). Salt may be available in the wall itself, from biological processes (ammonium salts) or simply due to co-migration with infiltrating water. Due to changes in physical parameters (i.e., temperature or humidity) salts can precipitate on the exposed surfaces. The crystallisation on walls and wall paintings results in a destructive effect, leading to

material loss and destruction due to cracking and detachment of the walls (Saiz-Jimenez *et al.*, 2012; López-Miras *et al.*, 2013)

1.1.1. Mitigation approaches

The recent field, Science for Conservation, has developed following two main streams: i) the characterisation of the technique used by the artists, the analytical characterisation of the materials constituting the works of art and the chemical reactions involved in their degradation; ii) the search for new scientific methods for the restoration/conservation, allowing the safeguard of our Cultural Heritage for its transmission to future generations (Giorgi *et al.*, 2010).

In general, the restoration of a work of art consists in:

1. Cleaning, which is a transient treatment, meant to remove the materials not originally belonging to the work of art;
2. Consolidation, which is a durable intervention that should remediate, prevent, or slow down further degradation due to aging or external agents (Giorgi *et al.*, 2010).

According to these purposes, during several years the conservation and restoration process was done without taken into account the presence of microbiological contamination in cultural heritage artefacts. In fact, many of the restoration procedures were short term processes, providing to microorganism all the nutrients needed for their development and proliferation. Thus, several efforts have been made to solve this problem.

In order to control the biodeterioration process the most suitable methods and products must be used. With regard to the principles and the nature of the means employed, biodeterioration control methods can be classified as: mechanical, physical, biological or biochemical. However, the chemical methods based on active principles in

solution are the most frequently applied (either as wide-spectrum active principles, or more specific, narrow-spectrum biocides such fungicides, algaecides, herbicides, insecticides and repellents for birds) (Allsopp *et al.*, 2004).

a) Mechanical Methods

The application of irradiation treatment for microbial elimination and cultural heritage artefacts protection has been used in several studies.

All the techniques described as "mechanical" have in common the method of displacing the biodeteriogens: physical removal. It can be carried out by hand or with tools such as scalpels, spatulas, scrapers, air abrasive or vacuum cleaners (Figure I-2A)

Although they were frequently used in the past, these methods do not produce lasting results and the eradication of contaminants could do not completely stop its vegetative activity. Moreover, the use of mechanical methods can damage the substrate, even if they have the advantage to avoid the addition of any substance that might cause further deterioration (Savulescu and Ionita, 1971).

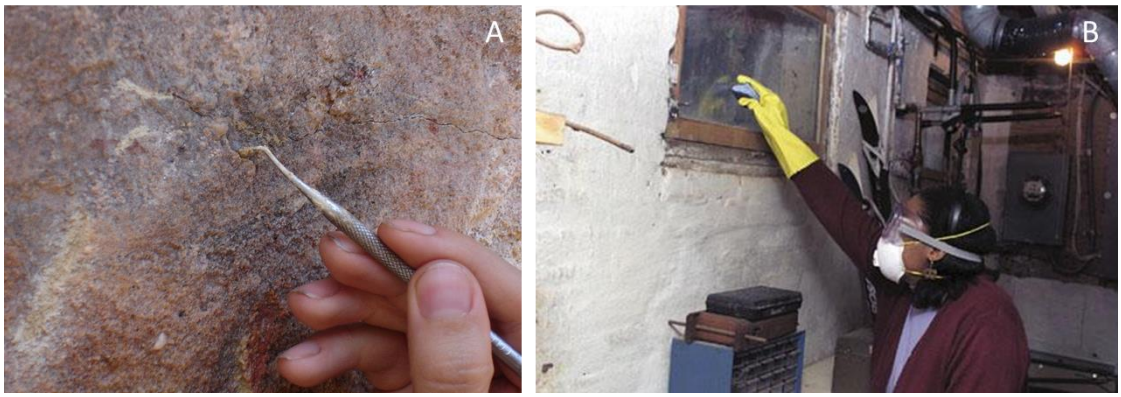


Figure I-2: Work of preventive cultural heritage conservation. A- Application of mechanical methods in a rock wall with prehistoric paintings at the Serra da Capivara, National Park, Brasil. B- Conservator-restorer cleaning mold with chemical toxic biocide.
(Adapted from http://www.wikiwand.com/en/Conservation-restoration_of_cultural_heritage and https://www.epa.gov/mold/brief-guide-mold-moisture-and-your-home)

b) Physical Methods

The physical methods used in some mitigation approaches are: ultraviolet radiation (UV), gamma-radiation, low frequency electrical systems, heat, deep-freeze temperatures, ultrasonic, titanium dioxide and laser techniques (Tiano, 2002; Scheerer *et al.*, 2009). Ultraviolet radiation has been used especially against bacteria, algae and fungi colonising of renders and plasters, however its handling can be dangerous and could cause injuries to the conservator-restorer. Gamma-radiation has been used for sterilising microflora and killing insects, especially on organic materials such as paper, parchment and wood. However, gamma irradiation does not have a long-lasting effect and can promote possible deterioration of the object to preserve (Abdel-Haliem *et al.*, 2013).

High-frequency can be used to kill wood-destroying insects (Anobiidae), but only in the absence of metals (Van Der Molen *et al.*, 1980; Cutler *et al.*, 2013). Low-frequency electrical current systems have recently been used to keep birds from roosting on monuments (Caneva *et al.*, 2008). This method is completely harmless for animals and humans. Heat (dry or wet) is used in the disinfestations and disinfections of organic materials (Gaylarde *et al.*, 2011).

Titanium dioxide is a photo-catalytic nanoparticle with antibacterial and antifungal abilities due to the production of reactive redox species (hydroxyl radicals, superoxide anions and hydrogen peroxide) which induce damages in the cell membrane and can inactivate a wide range of organisms like bacteria, viruses, fungi and algae. Titanium dioxide was proposed for preventing biodeterioration of mortars in cultural heritage buildings such as *Palacio da Pena* (Sintra, Portugal). However, despite these good indications it is necessary to take into account risks to humans as well as for paint materials because these particles are not as well studied neither their effect (De Filpo *et al.*, 2013).

Also, the use of laser techniques has been emergent in the last few years, due to their promising results not only for diagnostic but also for restoration procedures. Laser ablation takes place when a pulse of laser radiation is absorbed at the surface of a material, determining a sudden transition of its solid phase to another phase (gas, vapor, plasma). The sudden phase transition is mainly due to photothermal effects rising the temperature of the material up to a hot vapor that expands quickly in the surroundings, producing a material removal. However, this technique still present some inconvenient because do not completely remove the microorganism and could contribute for the deterioration of artworks (Salimbeni, 2006).

c) Biological and Biochemical Methods

The application of biological means is based on the existence of parasitic or antagonistic organisms of the biodeteriogens. Bacteria, insects and bacteriophage might be used as predators species and introduced on the environment in order to eliminate the coloniser microorganism (Caneva *et al.*, 2008).

The group of biochemical methods encompass biodeterioration control systems by the use of chemical compounds of biological origin. Antibiotics, enzymes and pheromones are the substances most commonly used (Ranalli *et al.*, 2005; Webster and May, 2006; Roig *et al.*, 2013)

d) Chemical Methods

Many organic and inorganic compounds have been used as biocide agents, to eliminate the biodeteriogens from cultural objects (Figure I-2B). The chemical biocides are classified in different ways depending on their chemical nature (e.g., organic or inorganic) or on the target species.

Pesticides, for example, are commonly used due to their biocide action with specific toxicity for the species to be eliminated. However, a problem associated with the use of pesticides is the persistence of the product in the soil or water. This issue is especially prevalent with herbicides that are applied or dispersed in external environments, with high risk for soil and water contamination (Blazquez *et al.*, 2000; Moreau *et al.*, 2008; Young *et al.*, 2008; Camara *et al.*, 2011).

Disinfectants constituted another example of biocides commonly used in conservation treatment. These can destroy vegetative forms but are not always effective against survival resistant or quiescent phase structures such as bacterial spores, fungal conidia and insect eggs. In addition to these biocides, other constituents, such as additives to improve the effectiveness of the product or to facilitate its application, are present in the chemical formulation. These are called co-formulants and could have negative effects on the objects to be treated (Nugari and Salvadori, 2003; Fonseca *et al.*, 2010; De los Ríos *et al.*, 2012).

1.1.2. Biocides treatment

According to the European Commission's Biocidal Product Directive 98/8/EC,1 (BPD) (http://ec.europa.eu/environment/biocides/index.htm) biocides can be divided into four main groups – disinfectant, preservative, pest control and other biocidal products – and further classified into 23 product types, including approximately 955 substances and 372 notified substances (Ashraf *et al.*, 2014). However, the choice of an appropriate biocide in Europe is limited by the single Directive previously cited. There are fundamental requirements for industrial biocides suitable for protection of materials like: effective antimicrobial activity, economical feasibility, very low human toxicity and compatibility with the environment (Singer *et al.*, 2010).

Although the number of chemical classes with biocide activity includes a wide variety of compounds, such as alcohols, aldehydes, phenols, acids, acid esters, amides,

carbamates, dibenzamidines, pyridines, azoles, heterocyclics, activated halogen compounds, surface active agents, organometallics and oxidising agents (Table I-1), the number of products suitable for cultural heritage is comparatively limited. This is due to the fact that only a small number of biocides have been tested with respect to their compatibility with historic materials, and only a very few studies exist about their long term effects (possible promotion of colour alterations or degradation products appearance) (Nittérus, 2000; Paulus, 2005; Sterflinger and Piñar, 2013). Despite the well-established short-term biocides efficiency, the removal of the microbial community may give rise to a new succession of microorganisms, which may be more damaging than the old microbial surface populations (Singer *et al.*, 2010).

Table I-1: Chemical biocides used in artworks treatment.

Biocide	Class/ Active principle	Action form	Reference
Preventol R50 **New-Des**	Ammonium quaternary compounds/ Na and Ca hypochlorite	Active transport and membrane integrity disruption	(Paulus, 2005; Nugari *et al.*, 2009; Maxim *et al.*, 2012; Silva *et al.*, 2016)
Diuron **Karmex**	Urea derivatives/ aromatic halide	Photosynthetic process blocking	(Blazquez *et al.*, 2000; Rosado *et al.*, 2014)
Wikamol Murosol	Organometallics/ Tributyltin oxide	Metabolism inhibitor	(Rosado *et al.*, 2014)
Igran 500FW	Triazines/ Terbutryn	Photosynthesis inhibition; electron transport alterations	(Fernandes, 2006; Maxim *et al.*, 2012)
Panacide	Chlorinated Phenol/ Dichlorophene	Clearing of intestinal contents increased by veterinary fungicide	(Silva *et al.*, 2016)

Against microbial recolonisation, some studies have suggested that the combined application of hydrophobic compounds and biocides is more effective than only of the biocide. The application can be done in a single step when the water-repellent and the biocide are mixed together, or in two steps when the biocide is applied before or after the water-repellent (Urzì and De Leo, 2007; Moreau *et al.*, 2008).

Therefore, the approach to control biodeterioration must be polyphasic and interdisciplinary, and must consider the history and condition of the artefact as well as the physical and chemical damaging factors (Scheerer *et al.*, 2009).

However, the mode of action of antimicrobial biocides and the associated resistance mechanisms are generally poorly understood (Mavri and Možina, 2013). The historical view that antimicrobial biocides possess broad-spectrum activity has led to their false association with low-target specificity (Ashraf *et al.*, 2014).

Moreover, a substantial part of all biocidal products currently available on the market act through toxic mechanisms 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 (Singer *et al.*, 2010; Ashraf *et al.*, 2014).

For these reasons, the chemical biocides with high toxicity can jeopardizes the health of any human beings, especially those that can easily handle them. In fact, conservators-restorers are the most direct persons exposed to these hazards, but few data are available regarding their health (Varnai *et al.*, 2011). This enhance the problem of the inadequate consideration of health and safety risks of biocides use. Despite the duty of each biocide manufacturer to have a Safety Data Sheet with all the health risk implicit, the importance of Cultural Heritage for Europe are still stressed to be central (Caminiti *et al.*, 2016) due to the real problems in term of safety and health that chemical biocides can bring.

Therefore, green alternatives based on natural biocides or innocuous solutions with very low toxicity, environmental acceptability, easy and safe to handling and storage, are currently essential in order to irradiate the potential risk associated with chemical biocides used (Silva *et al.*, 2016). This goal can be achieved by proposing adequate alternative materials, products and methods of intervention instead of the traditional ones.

Therefore, developing proper remediation actions for microbiologically contaminated historic materials based on environmentally safe solution is of vital importance.

Recently, biological cleaning is recognised as being a viable alternative to traditional chemical treatments such as organic solvents or mechanical treatments. In fact, microorganisms can act as new bioagents for the recovery and conservation of artwork and historical architectural monuments. These innovative biological methods (biocleaning, bioconsolidation) is encouraged by the fact that only a few known microorganisms play a destructive role (causing deterioration) in the natural processes, while the majority of them are responsible for positive processes (Azevedo *et al.,* 2009; Roig *et al.*, 2013). Careful selection of the appropriate (not pathogenic) microorganisms with the requisite characteristics for the removal of undesirable substances (nitrates, sulfates, organic matter, etc.) is one of the first steps to be taken in formulating the best biorestoration strategy. Biotechnological approach have been able to resolve a range of problems on various artistic materials (including monumental stone, wall paintings, marble statues, etc.) and to combat diverse pathologies that affect artworks (such as the bioremoval of organic substances, black crusts, and mineral salts) by using different cultures of viable bacteria; such as sulphate-reducing bacteria like *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris;* nitrate-reducing bacteria like *Pseudomonas stutzeri*, and others (Cappitelli *et al.*, 2005; Cappitelli *et al.*, 2007; Bosch-Roig *et al.*, 2012; Bosch Roig *et al.*, 2013).

However, the use of *P. stutzeri* has only been reported in the removing of organic matter in wall paintings (Sorlini and Cappitelli, 2008; Ranalli *et al.*, 2009; Polo *et al.*, 2010). Studies have described the different ways to expose the artwork to the bacteria beginning from its immersion in a solution, followed by it direct application using delivery systems like cotton, Carbogel and multilayer systems (Alfano *et al.*, 2011). The reports further emphasise that the most efficient method to apply the bacteria is by using a

delivery system where it provides enough water for their survival but not too much for not to be able to produce damage in the artwork (Cappitelli *et al.*, 2007; Roig *et al.*, 2013).

Hence, the use of viable microorganisms in biocleaning treatment has given rise to a number of questions about the risks of these methods. Restorers, public and private sector restoration committees are asking the scientific community for more information about the safety of this new technology.

In this way, importance must be given to process monitorisation in order to confirm and validate the biocleaning process, developing at the same time suitable strategies for inspection of any new microbial interactions on biocleaned artworks. When possible, these should include adequate on-site technologies based on non- invasive tools to understand the potential risks on biocleaned tangible heritage and include physical-chemical, biological, and aesthetic analyses (Roig *et al.*, 2013).

Similarly, further research and clear demonstrations of the complete safety of biocleaning is of fundamental importance because this technology has a very significant role to play in the introduction and diffusion of a new approach to the application of human-friendly, environmentally- sustainable techniques and technologies for the conservation and restoration of heritage properties (Bosch-Roig and Ranalli, 2014).

1.2. Biosurfactants compounds – a natural solution

Due to the limitations related with the use of chemical biocides, such as those mentioned above, it is urgent to find new products and antimicrobial compounds based on natural sources. This will allow to replace the risks associated with the use of chemicals products, avoiding the hazards associated to chemical products application.

Some solutions such as water-based micelles or microemulsions, (Giorgi *et al.*, 2010) and essential oils (Rakotonirainy and Lavédrine, 2005; Stupar *et al.*, 2014), are used in the replacement of chemical methods for cultural heritage safeguard.

A few studies report the use of oil-in-water microemulsions and micellar solutions formulated to solubilise acrylic and vinyl polymers from works of art (mainly wall paintings) and monuments (stones) (Carretti *et al.*, 2003). The aging of acrylic and vinyl polymers, used extensively in the past decades in art conservation, promote a long term yellowing and serious degradation of the painted layers or in the stone surfaces, imposing the necessity to process to their removal from the artistic and architectonic surfaces (Giorgi *et al.*, 2010). However, these strategy do not consider the microorganism interference and the need to mitigate its proliferation.

Stupar *et al*. 2014 reported that *Origanum vulgare*, *Rosmarinus officinalis* and *Lavandula angustifolia* (Lamiaceae) essential oils presented antifungal activity against fungi isolated from stone and wooden substrata, but mainly against *A. niger* and *A. ochraceus* (Stupar *et al*., 2014). However, studies described that the potential use of essential oils like linalool as an alternative to chemical fungicide is difficult to assess due to their fungistatic action rather than fungicide, but its use may be beneficial as a complement to controlled environment measures in preventing fungal contamination in storage areas of cultural properties (Rakotonirainy and Lavédrine, 2005).

Therefore, the solution for this problem can be centred in the use of natural products based on new compounds produced by microorganisms metabolic mechanism, which can represent a vast potential source of compounds with antimicrobial properties. In fact, it can be an effective alternative solution for the traditional chemical biocides substitution and a new mitigation strategy for biodegradation/biodeterioration process.

Natural substances with antimicrobial action have been identified from a very wide range of sources, including plants, microorganisms and animals (Roig *et al.*, 2013).

In particular, biosurfactants are biological surface-active compounds which present environmentally friendly properties, such as low toxicity and high biodegradability. These compounds, largely produced by a wide variety of microorganisms with capacity to low the surface and interfacial tensions, constitute a potential alternative to extensively used chemical synthesised surfactants (Desai and

Banat, 1997; Das *et al.*, 2008a; Roongsawang *et al.*, 2010). For their unique characteristics, biosurfactants are emerging as a promising tool for their use in agricultural, food, pharmaceutical, cosmetics and oil production industries as well as in bioremediation technology.

The hydrophobic portion of these surfactants is commonly constituted by fatty acids (saturated, unsaturated, or hydroxylated), whereas the hydrophilic portion is usually composed of peptides or mono-, di-, or polysaccharides (Georgiou *et al.*, 1992; Roongsawang *et al.*, 2010). Due to the presence of hydrophobic and hydrophilic moieties within a single molecule, biosurfactants tend to migrate toward an interface with different degrees of polarity and hydrogen bonding, such as interface air/water or oil/water (Desai and Banat, 1997).

Production of effective lipopeptide biosurfactants (LPBSs) was first reported from Gram-positive *Bacillus subtilis* IAM1213 (Arima *et al.*, 1968). Since then, various types of LPBSs with significant surface activity and/or anti-microbial activity have been isolated from other *Bacillus* strains (Peypoux *et al.*, 1984; Grangemard *et al.*, 1999; Yakimov *et al.*, 1999). Gram-negative *Pseudomonas* spp. also produce a variety of LPBSs (Ui *et al.*, 1997; De Bruijn and Raaijmakers, 2009; Raaijmakers *et al.*, 2010; Roig *et al.*, 2013)

A wide range of structurally different biosurfactants has been identified, including glycolipids, lipopeptides, polysaccharides, proteins, lipoproteins or mixtures of the former (Muthusamy *et al.*, 2008; Raaijmakers *et al.*, 2010).

1.2.1. Lipopeptides production

Antimicrobial peptides are multifunctional compounds with multiple roles. Among them, lipopeptides (LPP) are small molecules that are formed by cyclic or short linear peptides linked with a lipid tail or other lipophilic molecules (Raaijmakers *et al.*, 2010; Franco, 2011; Mandal *et al.*, 2013b).

The first lipopeptide discovered with antimicrobial activity, named polymyxin A, was isolated in 1949 from the soil bacterium *Bacillus polymyxa* (Jones, 1949). However, its biosynthesis has been detected in several bacteria genera, mainly *Bacillus, Pseudomonas* and *Streptomyces*, as well as in some fungal strains. Recently, several new LPP have been detected, showing diverse surfactant, antimicrobial and cytotoxic activities (Raaijmakers *et al.*, 2010). Some of them have even reached a commercial antibiotic status, like daptomycin (Robbel and Marahiel, 2010), caspofungin (Ngai *et al.*, 2011), micafungin (Emiroglu, 2011) and anidulafungin (George and Reboli, 2012).

Currently, lipopeptide properties may lead to their applications in diverse areas of such as pharmaceutical and cosmetic industry (applied in dermatological products), and food production (as emulsifiers).

Several questions have been raised about the mechanisms of action of LPP. Several studies have shown that pore formation in membranes occurs after lipopeptide oligomer binding, some of which are Ca^{2+} dependent multimers (Scott *et al.*, 2007). These pores may cause transmembrane ion influxes, including Na^{+} and K^{+}, which can result in membrane disruption and cell death (Scott *et al.*, 2007; Mangoni and Shai, 2011).

Some investigations have showed that lipopeptides, essentially due to their chemical nature, can inhibit fungi cell wall formation (Schneider *et al.*, 2009; Schneider and Sahl, 2010). Specifically, echinocardins a cyclolipohexapeptides, act by specific and non-competitive inhibition of β-(1,3)- D-glucan synthase, an essential component of the fungal cell wall responsible to keep its structural integrity. The lack of β-(1,3)- D-glucan leads to cell wall deterioration and consequently cell wall to keep its structural integrity. The lack of β-(1,3)- D-glucan leads to cell wall deterioration and consequently cell death (Yao *et al.*, 2012). However, the membrane is not the only target of lipopeptides. For example, at low concentrations, a new lipopeptide from surfactin family, named as WH1 fungincan and produced by *Bacillus amyloliquefaciens*, cause apoptosis by binding to ATPase on the mitochondrial membrane (Qi *et al.*, 2010).

The described modes of action may confer to LPP high activity against multidrug-resistant microorganisms (Mangoni and Shai, 2011), in fact the occurrence of microbial resistance to lipopeptides is extremely rare (Sader *et al.*, 2011).

Among the bacterial antimicrobial compounds known, the cyclic lipopeptides of the surfactin, iturin and fengycin (or plipastatin) families have recognised potential applications in biotechnology and biopharmaceutical fields (Ongena and Jacques, 2008).

Iturin production seems to be restricted to *B. subtilis* and *B. amyloliquefaciens* (Bonmatin *et al.*, 2003; Caldeira *et al.*, 2011b). Surfactin or closely related variants, such as lichenysin have been isolated, however, from *B. coagulans* (Huszcza and Burczyk, 2006), *B. pumilus* and *B. licheniformis* (Peypoux *et al.*, 1999). Fengycin production was identified in *B. cereus* (Tsuge *et al.*, 1999) and *B. thuringiensis* (Tsuge *et al.*, 1999), besides *B. subtilis* (Jacques *et al.*, 1999) and *B. amyloliquefaciens* (Caldeira *et al.*, 2011b).

a) Surfactin Family

The surfactin family encompasses several structural variants but all members are heptapeptides interlinked with a β-hydroxy fatty acid to form a cyclic lactone ring structure (Peypoux *et al.*, 1999) (Table I-2).

Table I-2: Structural variants diversity within the surfatin lipopeptide family synthesised by *Bacillus* species.

Surfactin variants	**Amino acids sequence**
Esperin	L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu-COOH
Lichenysin	L-XL_1-L-XL_2-D-Leu-L-XL_4-L-Asp-D-Leu-L-XL_7
Pumilacidin	L-Glu-L-Leu-D-Leu-L-Leu-L-Asp-D-Leu-L-XP_7
Surfactin	L-Glu-L-XS_2-D-Leu-L-XS_4-L-Asp-D-Leu-L-XS_7

XL_1=Gln or Glu; XL_2=Leu or Ile; XL_4 and XL_7 = Val or Ile; XP_7 = Val or Ile; XS_2 = Val, Leu or Ile; XS_4 = Ala, Val, Leu or Ile; XS_7 = Val, Leu or Ile

Surfactins are powerful biosurfactants with exceptional emulsifying and foaming properties. Due to their amphiphilic nature (Figure I-3), surfactins can readily associate and tightly anchor into lipid layers. This allow them to interfere with biological membrane integrity in a dose-dependent manner.

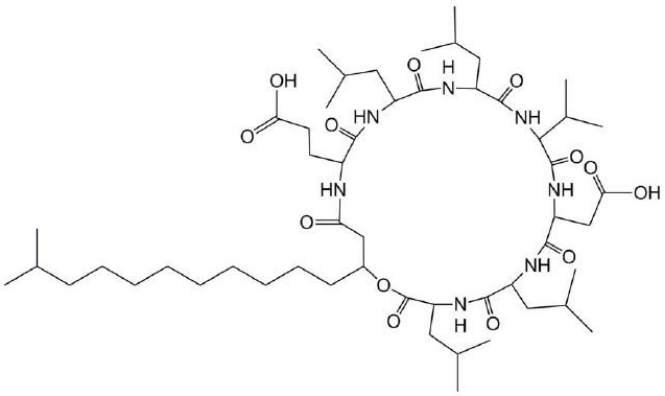


Figure I-3: Structure of a surfactin member synthesised by *Bacillus* species. Adapted from (Ongena and Jacques, 2008).

The destabilisation process of lipid bilayer membrane is facilitated by the three-dimensional form of the surfactin molecule featuring charged side chains protruding into the aqueous phase and apolar moieties reaching into the hydrophobic core of the membrane (Deleu *et al.*, 2003).

Due to their properties, surfactin exhibit anti-microbial, anti-tumour, anti-viral, and haemolytic properties (Dufour *et al.*, 2005; Ongena and Jacques, 2008; Roongsawang *et al.*, 2010).

b) Iturin Family

Iturin A and C, bacillomycin D, F, L and LC as well as mycosubtilin were described as the eight main variants within the iturin family (Table I-3).

Table I-3: Structural variants diversity within the iturin lipopeptide family synthesised by *Bacillus* species.

Iturin variants	Amino acids sequence
Bacillomycin D	L-Asn-L-Tyr-D-Asn-L-Pro-L-Glu-D-Ser-L-Thr
Bacillomycin F	L-Asn-L-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Thr
Bacillomycin L	L-Asp-L-Tyr-D-Asn-L-Ser-L-Gln-D-Ser-L-Thr
Bacillomycin LC	L-Asn-L-Tyr-D-Asn-L-Ser-L-Glu-D-Ser-L-Thr
Iturin A	L-Asn-L-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser
Iturin A_L	L-Asn-L-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser
Iturin C	L-Asp-L-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser
Mycosubtilin	L-Asn-L-Tyr-D-Asn-L-Gln-L-Pro-D-Ser-L-Asn

They are heptapeptides linked to a β-amino fatty acid chain with a length of 14 to 17 carbons (Figure I-4). Iturin A, in nature, is produced as a mixture of up to eight iturin isomers, A1-A8 (Ruangwong *et al.*, 2012a).

Due to their strong haemolytic action, the biological activity of iturins is different to surfactins: they display a strong *in vitro* antifungal action against a wide variety of yeast and fungi but only limited antibacterial (Caldeira *et al.*, 2008; Roongsawang *et al.*, 2010) and no antiviral activities (Moyne *et al.*, 2001).

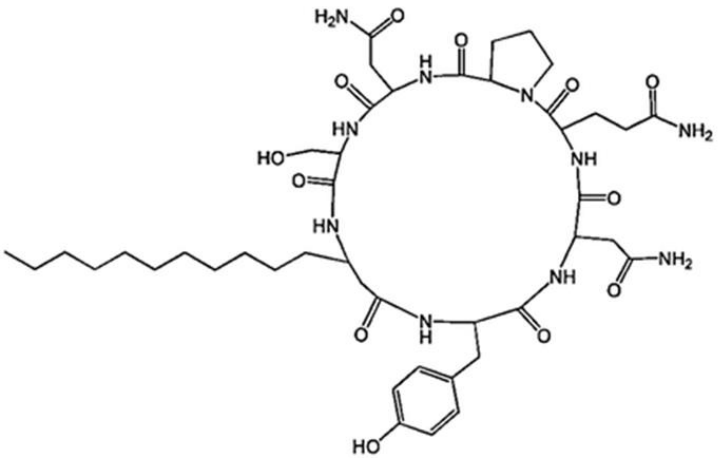


Figure I-4: Structure of an iturin member synthesised by *Bacillus* species. Adapted from (Ongena and Jacques, 2008).

The fungitoxicity of iturins almost certainly relies on the ability of these compounds to induce membrane permeabilisation. However, the underlying mechanism is based on osmotic perturbation owing to the formation of ion-conducting pores and not membrane disruption or solubilisation as occurs with surfactin. In this way these two families of compounds seems to possess different ways to act (Kim *et al.*, 2010a).

An ion-conducting pores formation in planar lipid bilayers has been explained as the result of the formation of aggregated structures in the lipid membrane. In fact, iturin A has been shown to be able to perturb the structure of membranes by modification of the curvature of phospholipid vesicles (Aranda *et al.*, 2005).

c) Fengycin family

Fengycins A and B are the third family of LPPs. They can also be called plipastatins (Table I-4).

Table I-4: Structural variants diversity within the fengycin lipopeptide family synthesised by *Bacillus* species.

Fengycin variants	Amino acids sequence
Fengycin A	L-Glu-D-Orn-D-Tyr-D-aThr-L-Glu-D-Ala-L-Pro-L-Gln-L-Tyr-L-Ile
Fengycin B	L-Glu-D-Orn-D-Tyr-D-aThr-L-Glu-D-Val-L-Pro-L-Gln-L-Tyr-L-Ile
Plipastatin A	L-Glu-D-Orn-L-Tyr-D-aThr-L-Glu-D-Ala-L-Pro-L-Gln-D-Tyr-L-Ile
Plipastatin B	L-Glu-D-Orn-L-Tyr-D-aThr-L-Glu-D-Val-L-Pro-L-Gln-D-Tyr-L-Ile

These lipodecapeptides are constituted by an internal lactone ring in the peptidic moiety bonded to a β-hydroxy fatty acid chain (C_{14} to C_{18}) that can be saturated or unsaturated (Figure I-5).

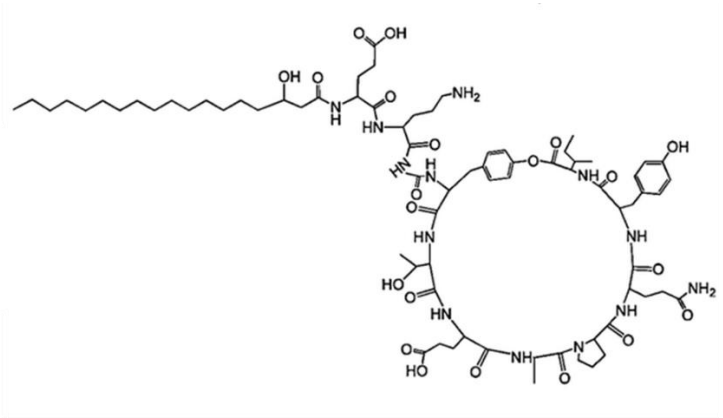


Figure I-5: Structure of fengycin member synthesised by *Bacillus* species. Adapted from (Ongena and Jacques, 2008).

Fengycins are less haemolytic than iturins and surfactins but they also exhibit a strong fungitoxic activity, specifically against filamentous fungi (Schneider *et al.*, 1999; Hu *et al.*, 2007; Kim *et al.*, 2010a). Mechanistically, the way of action of fengycins is less well known compared with those of other LPPs but they also readily interact with lipid layers, and to some level, retain the potential to alter cell membrane structure (packing) and permeability in a dose-dependent way (Deleu *et al.*, 2003).

In fact, lipopeptides are known to act in a synergistic manner as suggested by several studies on surfactin and iturin (Maget-Dana *et al.*, 1992), surfactin and fengycin (Ongena *et al.*, 2007) and iturin and fengycin (Romero *et al.*, 2007).

1.2.2. *Bacillus* sporulation

The efforts for production of new antibiotic are significant, in particular if the most of antibiotic biosynthesis are regulated by mechanisms shared with other starvation induced activities such as sporulation, genetic competence development and production of extracellular degradative enzymes (Stein, 2005).

Whereas the link between LPP production and *Bacillus* sporulation is not fully understood, it is associated, among other factors, to the stage of the culture. Due to the fact that their production are carried out at different growth phases during the culture incubation, the resting stage-sporulation induce the production of these lipopeptides (Caldeira *et al.*, 2008). In fact, some authors already report that *Bacillus* sp. sporulation phenomenon might occur due to a response to starvation that will eventually culminate in the production of antibiotic compounds such as lipopeptides (Marahier *et al.*, 1993; Piggot and Hilbert, 2004; Rahman *et al.*, 2006; Caldeira *et al.*, 2008; Jin *et al.*, 2015).

A wide range of bacteria use specialised differentiated cell types (i.e., spores) for dealing with starvation and surviving under harsh conditions (Stein, 2005). In fact, nutrient-limited *B. subtilis* cells (able to sporulated), release an endospore from the terminally differentiated, apoptotic mother cell. Endospores are almost certainly the longest surviving cells and they are formed by ancient (deeply-rooted) lineages of bacteria, the best known of which is *Bacillus* (Errington, 2003).

The endospore is formed by an unusual mechanism involving asymmetric cell division, followed by engulfment of the smaller cell (Prespore or Forespore) by its larger sibling (Mother cell or Sporangium) (Figure I-6).

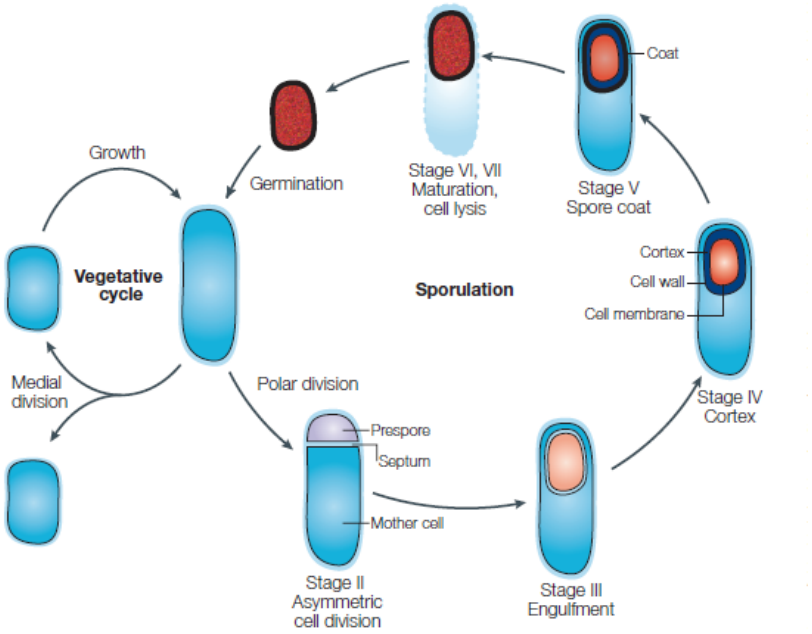


Figure I-6: The key stages of the sporulation cycle of *Bacillus subtilis*. Adapted from (Errington, 2003).

The success of endospore formation lies in the altruistic behaviour of the mother cell, which uses all of its resources to endow the prespore with resources, particularly protective layers. This maximises the chances of survival of the mature spore. Spores formation takes about 7 h at 37ºC and can survive treatments that rapidly and efficiently kill other bacterial forms, including high temperatures (even 100°C), ionising radiation, chemical solvents, detergents and hydrolytic enzymes. They can remain dormant for immense periods of time, perhaps even millions of years (Nicholson *et al.*, 2000; Errington, 2003).

As referred before, the main stimulus for sporulation is starvation. However, for this process to occur, a high density of cells needs to be present in the culture. No single nutritional effect acts as trigger. Rather, the cell has an extremely complex and sophisticated decision-making apparatus, which monitors a huge range of internal and external signals. The information is channelled through several separate regulatory systems, of which the most prominent component is an important transcriptional regulator called Spo0A. Spo0A synthesis is controlled transcriptionally, and the activity

of the protein is regulated by phosphorylation. Phosphorylated Spo0A is an essential positive regulator of sporulation, and it works by activating the transcription of several key sporulation-specific genes, particularly the spoIIA, spoIIE and spoIIG genes (Jiang *et al.*, 2000; Errington, 2003).

The other key positive regulator of sporulation is σH (Sigma factor) which interacts with core RNA polymerase. In interaction with Spo0A, σH act in regulatory pathways and overlap in ways that are not yet fully understand. Superimposed on this positive regulation are multiple negative regulators of transcription, such as codY, abrB and soj (Piggot and Hilbert, 2004).

During sporulation, the bacterial cell is dramatically reorganised to generate two daughter cells of very different size and fate, the smaller prespore and the larger mother cell. The nucleoids of the vegetative cell are remodelled into a continuous structure, the axial filament of chromatin, which extends the length of the cell (Figure I-7) (Piggot and Hilbert, 2004).

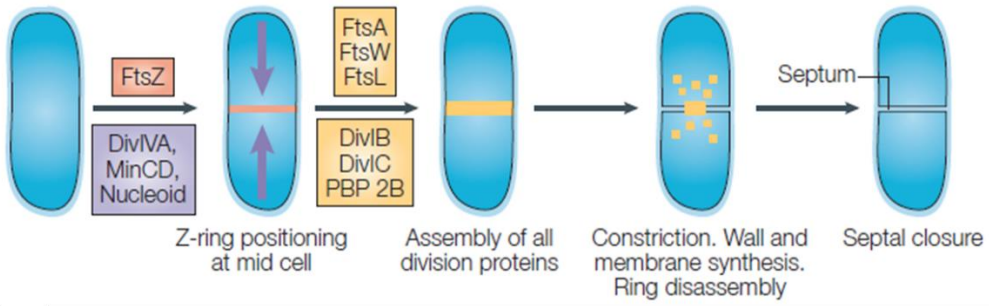


Figure I-7: Cell division during *Bacillus subtillis* growth. Adapted from (Errington, 2003).

RacA has been identified as a protein produced during sporulation that binds the chromosome and the polar division protein DivIVA, acting as a bridge connecting the two. In its absence axial filaments and an anucleate prespores are not formed. Concomitant with axial filament formation, the ring of the essential tubulin homolog FtsZ at midcell relocalises, via a helical intermediate, to sites near the cell pole and the

asymmetric division occurs at one of those sites. This relocalisation is triggered by a σ^H-dependent burst of FtsZ expression and the Spo0A-dependent expression of SpoIIE. After division, only the origin-proximal one-third of a chromosome is present in the prespore; the remainder is then transported through the septum by the DNA translocase SpoIIIE (Figure I-8) (Errington, 2003).

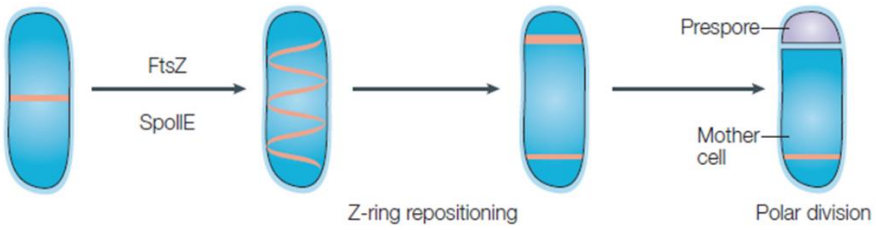


Figure I-8: Chromosome partitioning and *Bacillus subtillis* asymmetric division. Adapted from (Errington, 2003).

The transient genetic asymmetry between prespore and mother cell play a key role in the establishment of compartmentalised gene expression.

Early compartmentalised gene expression results in modification of the asymmetric septum. So, its peptidoglycan is removed and it begins to migrate around the prespore, fusing to release the prespore as a protoplast within the mother cell, a process known as engulfment.

After the asymmetric division and before a chromosome has completely partitioned into the prespore, σ^F becomes active exclusively in the prespore (Figure I-9). In the pre-divisional cell σ^F is held inactivate by the anti-s factor SpoIIAB; this inhibition is reversed by the anti-anti-s factor SpoIIAA. SpoIIAA is regulated by its phosphorylation state; it is inactive when phosphorylated by SpoIIAB (a kinase as well as an anti-s) and active when it is dephosphorylated by SpoIIE. How σ^F activation is linked to asymmetric division and limited to the prespore, remains under investigation. SpoIIE localizes asymmetric division sites and interacts with FtsZ; it has been proposed to 'sense'

asymmetric division and activate σ^F in response (Errington, 2003; Piggot and Hilbert, 2004).

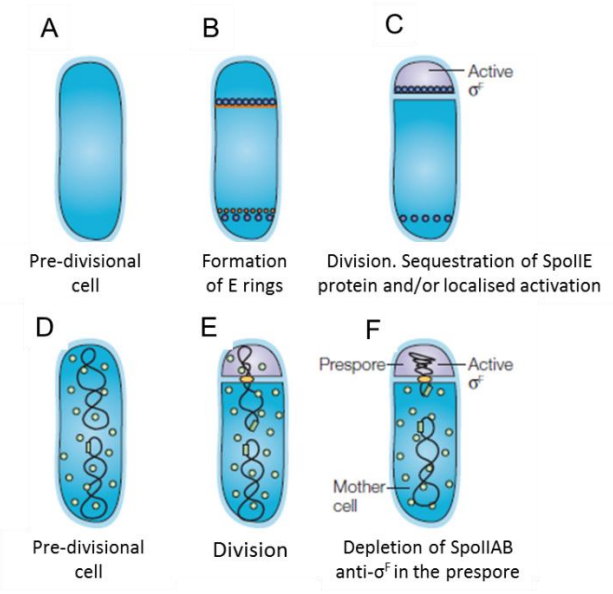


Figure I-9: *Bacillus* cell-specific activation of σ^F. Adapted from (Errington, 2003).

Activation of σ^F in the prespore is rapidly followed by activation of σ^E in the mother cell. On receipt of a signal (SpoIIR) from the prespore, the inactive membrane-bound precursor, pro- σ^E, is processed to an active state, most likely by the putative protease SpoIIGA.

Expression of the gene encoding pro-σ^E, spoIIGB, is greatly enhanced by the master response regulator Spo0A. In fact, Spo0A activity takes place in the mother cell following asymmetric division. As a consequence, the level of pro-σ^E becomes much higher in this compartment than in the prespore, contributing to compartmentalised σ^E activity.

σ^G is synthesised in the pre-engulfment prespore and held inactive before the completion of engulfment; it was previously thought that SpoIIAB was responsible for the inhibition. However, a mutant form of σ^G that is not bound by SpoIIAB does not become active in the prespore until engulfment is complete. Further, although SpoIIAB binds to and inhibits σ^G, the inhibition is reversed by dephosphorylated SpoIIAA, which is present

in the prespore before engulfment (Evans *et al.*, 2003). SpoIIAB-independent regulator(s) couple to σ^G activation in the prespore to the completion of engulfment; it may be that SpoIIAB fulfils a partly redundant role in preventing activation in the mother cell (Errington, 2003). The products of the spoIIIA and spoIIIJ loci are required for σ^G activation; whereas SpoIIIJ is normally produced vegetatively, it can support sporulation if only produced in the prespore. By contrast, spoIIIA is expressed only in the mother cell and switching it is site of expression to the prespore impaired spore formation. These results suggest that a signal is transmitted from one or more products of the spoIIIA operon to SpoIIIJ in the prespore to release σ^G from inhibition, although additional factors are likely to be involved (Piggot and Hilbert, 2004).

The late mother cell σ factor σ^k is synthesised as an inactive precursor. Genetic evidence indicated that pros-K is processed by the putative protease SpoIVFB, and that this reaction is inhibited by SpoIVFA and BofA. The roles of these proteins are beginning to be clarified; processing of pro- σ^k by SpoIVFB has been demonstrated in *E. coli* and found to be inhibited by BofA (Zhou and Kroos, 2004). SpoIVFA mediates this inhibition by bringing BofA and SpoIVFB together in a multimeric complex localised to the outer prespore membrane (Rudner and Losick, 2002). Protein localisation is also an area of interest. In contrast to the directed insertion that occurs in the inner prespore membrane (Rudner and Losick, 2002), SpoIVFB is initially inserted into both outer prespore and peripheral membranes non-specifically, and then is retained only in the outer prespore membrane by SpoIVFA, a process termed “diffusion and capture” (Rudner *et al.*, 2002). Processing of pro- σ^k requires a signal, SpoIVB, from the prespore. SpoIVB has been found to proteolyze SpoIVFA; if this reaction occurs it would separate SpoIVFB from its inhibitor BofA and thereby trigger processing (Piggot and Hilbert, 2004).

In general, components of the developing spore are synthesised in the compartment where they are needed. There is one striking exception, where a spore component appears to be made in the wrong compartment. Dipicolinic acid (DPA) is made in the mother cell by the products of σ^k -controlled spoVF locus (Piggot, 2002).

However, DPA (probably as a 1:1 complex with Ca^{2+}) is required in the spore, where it constitutes ~10% of the spore dry weight and is important for heat resistance (Paidhungat *et al.*, 2000). The products of the σ^G -directed spoVA locus are required for transport of DPA into the prespore (Tovar-Rojo *et al.*, 2002). It is not clear why the system is organized in this surprising way. A possible explanation is that DPA is secreted early in germination, and it may be that the same SpoVA complex is somehow required for this secretion, as well as for the prior DPA import into the prespore (Errington, 2003).

The centre of the spore undergoes marked changes in physicochemical properties as it develops. Low-molecular- weight proteins are synthesised in large amounts to coat the DNA, providing protection against several kinds of DNA damage. The same proteins are broken down during germination to provide a source of amino acids. Large amounts of dipicolinic acid are synthesized in the mother cell and taken up by the prespore, together with divalent cations (Ca^{2+}) which leads to the dehydration and mineralisation of the spore. Meanwhile, the spore cortex, a modified cell wall, is synthesized outside the spore protoplast membrane. Finally, a multilayered proteinaceous coat is assembled outside the cortex. In some spore formers, an exosporium is present as an extreme outer layer (Nicholson *et al.*, 2000; Errington, 2003).

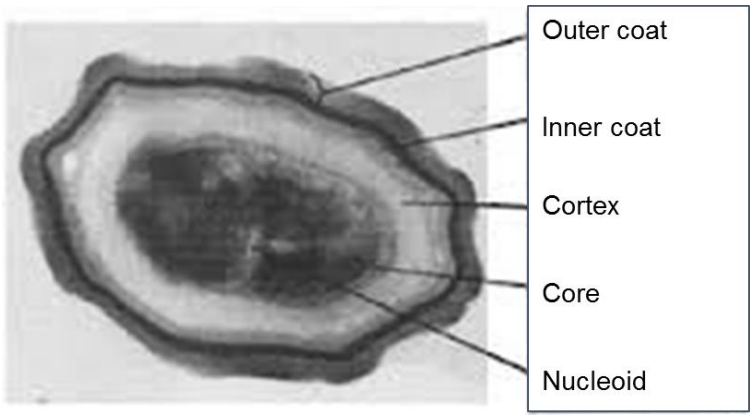


Figure I-10: Cross-section of a spore of *Bacillus subtilis*, showing the cortex and coat layers surrounding the core (dark central area). The spore is 1.2 microns across, about 100 times smaller than the width of a human hair. Adapted from (Nicholson *et al.*, 2000).

As the spore is being constructed, its ability to respond to specific germinants, to shed its protective layers and to rehydrate and resume vegetative growth are also built into the structure (Rahman *et al.*, 2006; Caldeira *et al.*, 2008).

The regulation of most of these activities is underpinned by the later prespore and mother-cell sigma factors, which work together with the auxiliary regulators described above (Errington, 2003).

1.2.3. Lipopeptides biosynthesis

LPPs are synthesised by large nonribosomal peptide synthetases (NRPSs) through a thiotemplate process (Figure I-11). NRPSs have a modular structure in which each module, of approximately 1000 amino acids, catalysed the incorporation of one amino acid in the peptide product (Stein, 2005).

Members of the iturin family, are synthesised by polyketide synthases (PKS) as well as fatty acid synthetases, yielding a hydrid PKS/NRPS biosynthetic template (Duitman *et al.*, 1999; Tsuge *et al.*, 2001).

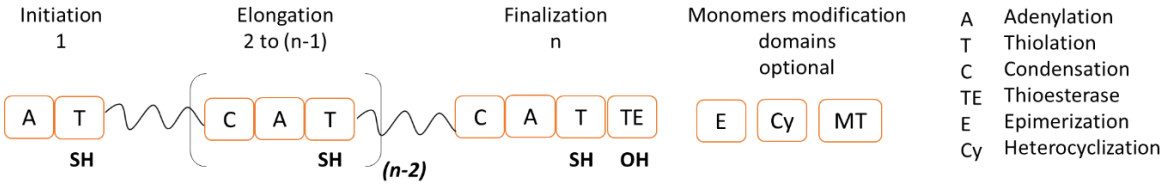


Figure I-11: Organisational structure of peptide synthetases located outside of the ribosome. Adapted from (Kohli and Walsh, 2003).

The NRPSs, which are composed of modularly arranged catalytic domains, catalyse all necessary steps in peptide biosynthesis including the selection and ordered condensation of amino acid residues. Therefore, modules can be subdivided into

adenylation (A), thiolation (T) and condensation (C) domains (Stein, 2005; Gross and Loper, 2009; Roongsawang *et al.*, 2010).

In most cases, the number and order of the NRPS modules are collinear to the amino acid sequence of the peptide (collinearity rule). The different modules can be subdivided in initiation and elongation modules. Initiation modules typically consist of an adenylation domain (550 amino acid residues), responsible for amino acid selection and activation, and a thiolation domain (80 amino acid residues) responsible for thioesterification of the activated amino acid (Moyne *et al.*, 2004).

However, in LPP biosynthesis the first module also contains a condensation domain (450 amino acid residues). This C_1 domain catalyses N-acylation of the first amino acid of the molecule, thereby linking the lipid moiety to the oligopeptide (Roongsawang *et al.*, 2003). Elongation modules contain A, T and C domains, in which the C domain catalyses peptide bond formation between 2 amino acid. Together, these domains generate a lipopeptide, which is cleaved at the end of the assembly line by a thioesterase (TE). In general cases, the TE domains catalyse the cyclization of the mature peptide product rather than its hydrolytic cleavage, resulting in the release of a cyclic LPP (cLPP) (Schwarzer *et al.*, 2001; Raaijmakers *et al.*, 2010)

As the corresponding linear forms of cLPPs are in many cases biologically less active, it was postulated that cyclization reduces conformational freedom and provides stabilisation of the compound. This is necessary for interaction with the biological target. A second type of TE is also necessary for the functioning of these NRPS systems, as repair enzyme (Sieber and Marahiel, 2003).

NRPSs may harbour epimerization (E) domains that determine the configuration (L or D) of the incorporated amino acid. For example, the surfactin biosynthetic template in *Bacillus* contains two E domains that are responsible for the incorporation of two D-Leu residues (Sieber and Marahiel, 2003; Muthusamy *et al.*, 2008). In contrast to *Bacillus*, no E domains have been found so far in LPP biosynthetic templates of *Pseudomonas* species (Roongsawang *et al.*, 2003).

Analyses of the metabolite profiles of *Bacillus* species show that single strains can simultaneously produce several LPP families, but also multiple structural analogues of one particular LPP (Bonmatin *et al.*, 2003)

The fact that LPP-biosynthesis mutants do not produce the main LPP or any of its derivatives indicate that these analogues are the result of the flexibility in amino acid selection and activation by the A domains. Substrate flexibility of A domains is a common phenomenon in nonribosomal peptide synthesis and, instead of being considered a "mistake" of the A domains, this may have biological functions for the producing strain.

Surfactins has shown as a small structural changes in the peptide or lipid tail not only affected physicochemical properties such as micellisation, oil displacement or reduction of surface tension, but also their interaction with phospholipid bilayers and antimicrobial activities (Peypoux *et al.*, 1999; de Bruijn *et al.*, 2007).

However, such flexibility of the A domains is not always the rule as exemplified for iturins: iturin A, mycosubtilin and bacillomycins produced by different strains of *B. subtilis* differ in their amino acid composition but, for certain strains, peptidic variants appear to be lacking (Bonmatin *et al.*, 2003).

The operons that encode surfactin (Cosmina *et al.*, 1993), plipastatin-fengycin (Lin *et al.*, 1999), mycosubtilin and bacillomycin (Duitman *et al.*, 1999), which is a member of the iturin A group, have been sequenced and characterised (Tsuge *et al.*, 2001; Moyne *et al.*, 2004; Tsuge *et al.*, 2005).

The results shown that surfactin operon have four "Open Reading Frame" (ORFs) namely srf A, srf B, srf C and srf D, which correspond to the surfactin synthetase enzyme. It also contains sfp gene encoding phosphopantetheinyl transferase enzyme, required for posttranscriptional modification of surfactin (Das *et al.*, 2008b). Specifically, the three loci, srfA, srfB and srfp, have been shown to be essential for surfactin production; srfA and sfp are genetically linked but encoded by segregating characters, while srfB maps elsewhere and has been shown to be identical to the comA gene. ComA is a member of the two component signal transduction systems which functions as a transcriptional

activator on the srfA promoter, although direct inter-action has not been demonstrated (Cosmina *et al.*, 1993).

Three distinct operons that belong to the iturin group have been cloned and sequenced, the mycosubtilin, the iturin A and the bacillomycin D operon (Tsuge *et al.*, 2005). All of these operons are composed of a putative transcriptional unit with four genes: i) one small gene that encodes malonyl-coenzyme A transferase and is probably responsible for α-amino acid synthesis; and ii) three large genes that encode large template enzymes for the synthesis of peptides with defined sequences and chirality (Sader *et al.*, 2011).

In the case of mycosubtilin operon, it contains additionally a fatty acid enzymatic domain along with a peptide enzymatic domain to form a hybrid synthetase in the second ORF mycA. Another member of the iturin family, the iturin A synthetase operon, has been completely sequenced and shown to share the same organisation as the mycosubtilin operon and is composed of four ORFs itu D, itu A, itu B and itu C transcribing in the same direction (Moyne *et al.*, 2001; Tsuge *et al.*, 2001) (Figure I-12).

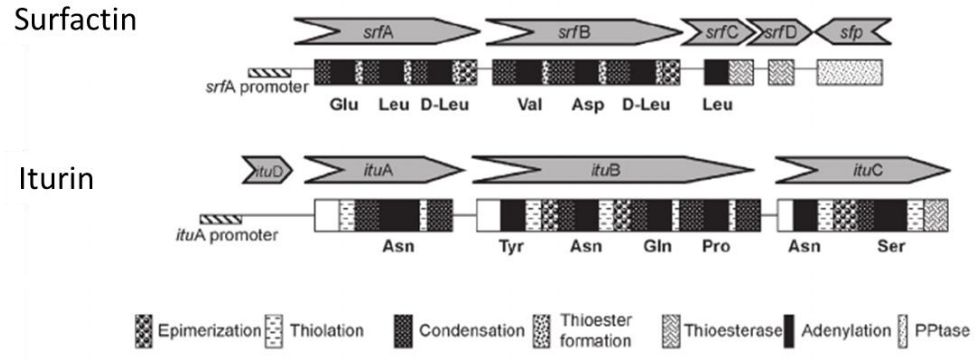


Figure I-12: Structural organisation of the genes encoding surfactin and iturin biosurfactant synthetases family. Adapted from (Das *et al.*, 2008b).

Relatively to fengycin, this LPP operon contains five NRPS subunits: fenC (287 kDa), fenD (290 kDa), fenE (286 kDa), fenA (406 kDa), and fenB (146 kDa). Like surfA, fengycin is also composed of an N-acyl domain at the N-terminus of fenC, conventional

E-domains, and a typical type I Te-domain. Fengycin assembles forming a co-linear chain ordered as fenC-fenD-fenE-fenA-fenB (Roongsawang *et al.*, 2010), which is represented in Figure I-13.

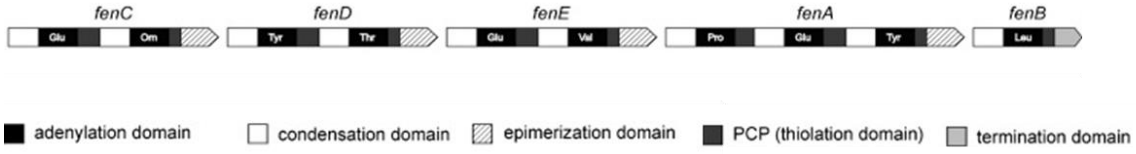


Figure I-13: Structural organisation of the genes encoding fengycin synthetases family. Adapted from (Stein *et al.*, 2005).

1.2.4. Lipopeptide detection and characterisation

Multiple qualitative tests are available to detect LPP, including drop collapse and haemolysis assays, tensiometric and spectrophotometric analysis. Although these methods are useful for high throughput screening of isolates, the obtained results should be carefully interpreted. The identity of the LPP requires confirmation by analytical–chemical techniques, such as Thin-layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Mass Spectrometry (MS), chiral Gas Chromatography (GC), Crystallography, Fourier transform IR spectroscopy (FTIR) and/or Nuclear Magnetic Resonance (NMR) (Raaijmakers *et al.*, 2006).

The analytical efficiency of these technologies was confirmed in previous studies where the entire spectrum of *Bacillus* LPP and their biosynthetic intermediates were detected and identified in cells culture (Debois *et al.*, 2008; Nihorimbere *et al.*, 2009).

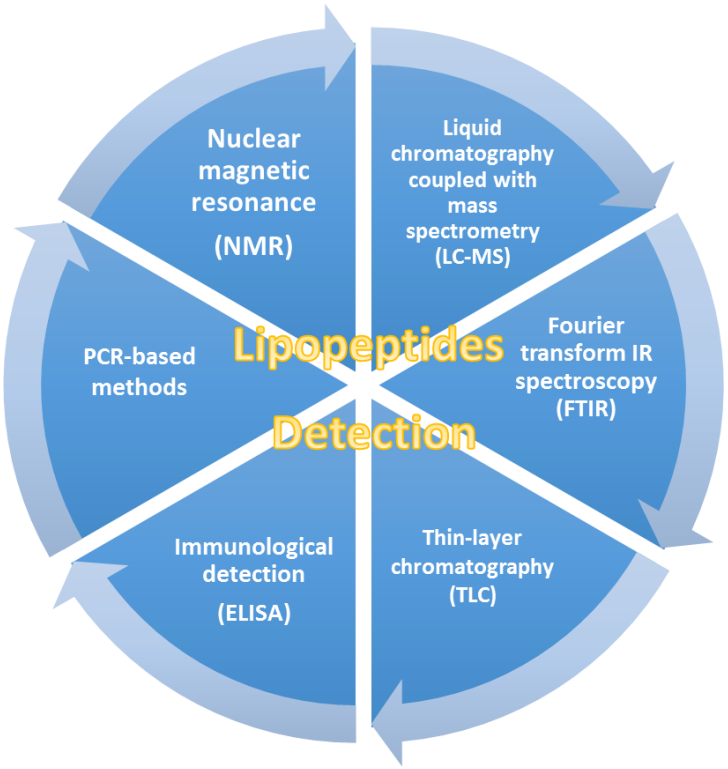


Figure I-14: Multianalytical approaches to *Bacillus* lipopeptides detection.

Immunological detection, despite is still in its beginning, were also proposed as a sensitive approach to detect and quantify LPP *in situ*, especially in plant-associated environments (where relatively low amounts of LPP are produced and where plant-derived compounds may interfere with chemical detection) (Zhao *et al.*, 2001). Specific antibodies in Enzyme-Linked Immunosorbent Assay (ELISA) assays will be highly instrumental as a supplementary approach to study the localization of LPP in complex environments or to monitor their fate and stability after its application to plant tissues or other habitats (Raaijmakers *et al.*, 2010).

For molecular detection of LPP producers, PCR-based methods were developed in *Bacillus* strains. PCR primers were designed against specific sequences in various LPP biosynthesis genes and successfully used to identify soil isolates with antifungal activity (Joshi and Gardener, 2006; Hsieh *et al.*, 2008; Cao *et al.*, 2012; Mohkam *et al.*, 2016). In fact, the use of specific primers for LPP biosynthesis genes, resulted in more specific detection of LPP producers with a positive result for strains tested (Sorensen *et*

al., 1998). However, in some cases the high level of sequence similarity between different LPP biosynthesis genes makes difficult to design specific primers for detection of strains producing a particular lipopeptide.

Nevertheless, the analytical technique most commonly used is mass spectrometry coupled with liquid chromatography (LC–MS) (Pathak *et al.*, 2014). This technique enables the fingerprinting of novel bioactive metabolites directly from crude extracts prepared from natural sources. Its application has several advantages as it allows screening and identification of the metabolites without their previous isolation, even when they are present at low concentrations. This results in time, money and energy savings.

Recently, NMR spectroscopy was also used as a new tool for lipopeptide characterisation (Scott *et al.*, 2007; Pereira *et al.*, 2013; Tareq *et al.*, 2014). NMR spectroscopy evolved primarily as a tool for the characterisation of pure compounds or simple well-defined mixtures. However, various strategies for the NMR spectroscopic identification of compounds from complex mixtures have been recently developed. The use of NMR spectroscopy for the analysis of complex mixtures opens up new perspectives and enables new lines of inquiry in both natural products chemistry and metabolomics (Bleicher *et al.*, 1998; Harner *et al.*, 2013; Herrmann, 2014; Simmler *et al.*, 2014). Following the initial observation that the resonance frequency of a nucleus is influenced by its chemical environment, and that the fine structure of a resonance could be influenced by other nucleus through intervening chemical bonds, quickly the potential of NMR spectroscopic tools was recognise. As a result, NMR became one of the most important spectroscopic tools of organic chemistry. Combining NMR spectroscopic with mass spectrometric analyses proved to be particularly useful, with MS providing information about molecular weight and atomic composition of LPP, and NMR contributing information about chemical environment and, importantly, connectivity and spatial configuration (Mander and Liu, 2010).

The basis of this PhD research project has been the development of integrated studies that combine a selection of promising bacterial strains with antifungal potential, the detection and characterisation of the bioactive compounds produced by those strains, the study of bacteria cells physiology and, finally, toxicological assessment and antifungal efficiency of the bioactive compounds in cultural heritage context. This research was developed using different methodology combined each one for a final unique propose: the development of a novel biocide for cultural heritage.

The organisation of this PhD thesis is established into six chapters; an introductory chapter, four chapters dedicated to the results and discussion, being most of them published in scientific papers, and a final chapter presenting the main conclusions obtained during this research.

Chapter I describes general aspects related to the Cultural Heritage Biodeterioration/Biodegradation phenomena, giving an overview about the importance of microorganisms in the artworks decay, the different methods to promote artworks longevity and the aspects related to the use of chemical biocides, based on an extensive bibliographical research. Also, it was explore the molecular and physiologic mechanism behind the lipopeptide production by *Bacillus* strains and their importance in artworks rehabilitation.

Chapter II describes the way to select and identify bacterial strains with potential to produce compounds with antifungal capacity against biodeteriogenic fungal strains from artworks with biodegradation/biodeterioration signs.

In Chapter III are presented a methodological basis to detect and characterise the bioactive compounds produced by selected bacterial strains.

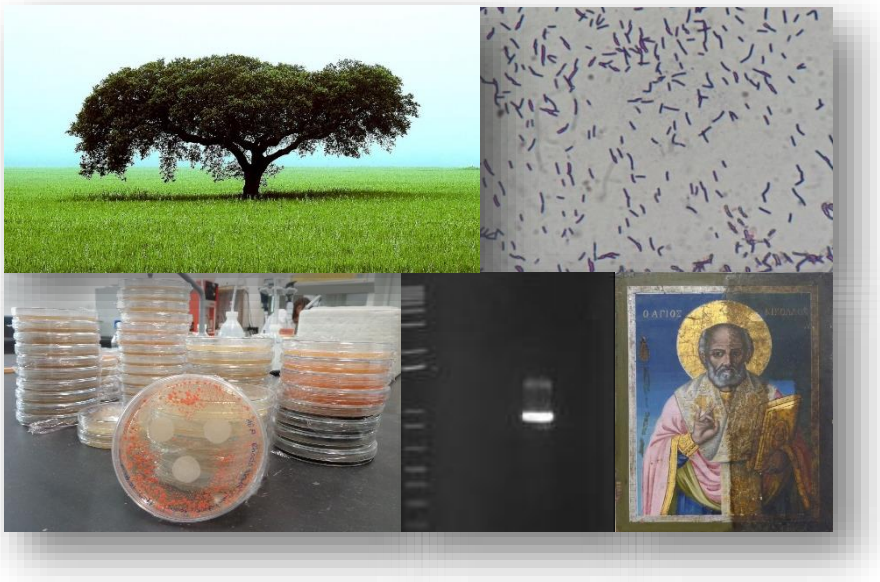
Chapter IV intend to clarify the physiological phenomena underneath the bioactive compounds production, using a ground-breaking methodology for the physiological response of bacteria strain to nutrient starvation and biological active compounds production monitorisation.

Chapter V is focused on the toxicological evaluation, using two different biological models, of the new bioactive compounds produced, by the selected bacterial strains. Also, simulation studies were performed in the presence of the new compounds in order to explore their real life efficiency and possible influence on the structure of mural painting samples.

Final considerations and future perspectives are presented in Chapter VI, emphasising the advantages of the strategies outlined.

CHAPTER II

Microorganisms producers of Lipopeptides



Some results of this chapter were published in the following scientific publication:

Silva M., Rosado T., Teixeira, D., Candeias A., Caldeira A. T. (2017). Green mitigation strategy for Cultural Heritage: Bacterial potential for biocide production. Environmental Science and Pollution Research, 24:4871–4881

1. Overview

Different structurally biosurfactants, including glycolipids, lipoproteins, polysaccharides, proteins and lipopeptides are known to be produced by a wide variety of microorganisms. Some *Bacillus* strains are known to produce lipopeptides (LPP) included in the surfactin, the fengycin and the iturin group, that are synthesised nonribosomally by a mega-peptide synthetase unit (NRPS), which is composed by several cooperating multifunctional modules.

The characterisation, identification and evaluation of the bacterial strains responsible for the antibiotic potential as well as the detection of the presence of oligonucleotide regions responsible for the LPP biosynthesis was performed.

Therefore, this chapter intend to develop a molecular procedure to the selection of LPP producer strains in view of LPP bioactive compounds production with the last aim of applying them as green natural biocides in biodegraded cultural heritage context.

The results reveal four potential bacteria strains, producers of antifungal compounds. These bacteria exhibit the same morphological and biochemical characteristics, possible belonging to the genera *Bacillus*. Sequence analysis of the iturin operon revealed four ORFs with the structural organisation of the peptide synthetases. The methodology applied, involving PCR and Real-time PCR proved to be valuable for quick identification of iturin -producing strains, constituting an effective approach for identification and selection of lipopeptides producer strains.

2. Introduction

Biosurfactants are biological surface-active compounds produced by a variety of microorganisms (Dehghan-Noude *et al.*, 2005; Das *et al.*, 2008a; Cao *et al.*, 2009). A wide range of structurally different biosurfactants have been identified, including glycolipids, lipoproteins, polysaccharides, proteins and lipopeptides (Souto *et al.*, 2004; Roongsawang *et al.*, 2010). Specifically, the lipopeptides are commonly made up of a hydrophobic portion composed by fatty acids (saturated, unsaturated, or hydroxylated), that are linked to a short linear or cyclic oligopeptide that mark the hydrophilic portion of the molecule (Raaijmakers *et al.*, 2010). On the basis of the structural relationships, the lipopeptides that have been identified as products of the metabolism of some *Bacillus* strains are generally classified into three groups: the surfactin (Yao *et al.*, 2003; Mikkola *et al.*, 2004), the fengycin (Hu *et al.*, 2007; Arrebola *et al.*, 2010) and the iturin group (Moyne *et al.*, 2001; Kim *et al.*, 2010a; Mandal *et al.*, 2013a).

The members of the surfactin and fengycin groups are composed of one β-hydroxy fatty acid and 7 and 10 α-amino acids, respectively, while the members of the iturin group consist of one β -amino fatty acid and 7 α -amino acids. The presence of the β-amino fatty acid is the most striking characteristic of the iturin A family and distinguishes it from the other two families (Tsuge *et al.*, 2001).

These compounds have been received significant attention due to their antimicrobial and surfactant properties, especially the nonribosomally synthesised lipopeptides surfactin, iturin and fengycin (Caldeira *et al.*, 2011a). Whereas the mechanism of biosynthesis of these lipopeptides is mostly conserved, they have diverse structures (Zhao *et al.*, 2013). The lipopeptides are synthesised nonribosomally by a mega-peptide synthetase unit (NRPS) which is composed by several cooperating multifunctional modules, each one capable of performing one cycle of peptide elongation (Roongsawang *et al.*, 2010).

NRPSs have a modular structure in which each module (of approximately 1000 amino acids) catalysed the incorporation of one amino acid in the peptide product. The order of the modules corresponds to the order in which amino acids are incorporated in the peptide product (Roongsawang *et al.*, 2010). Modules can be further subdivided into adenylation (A), thiolation (T) and condensation (C) domains (Figure II-1). The adenylation domain recognises and activates the amino acid, which is then linked to the 4' phosphopanthetheinyl cofactor attached to the thiolation domain. Subsequently, the condensation domain located between two activating domains binds two consecutive amino acids during the elongation of the peptide product (Moyne *et al.*, 2004; Ramarathnam *et al.*, 2007).

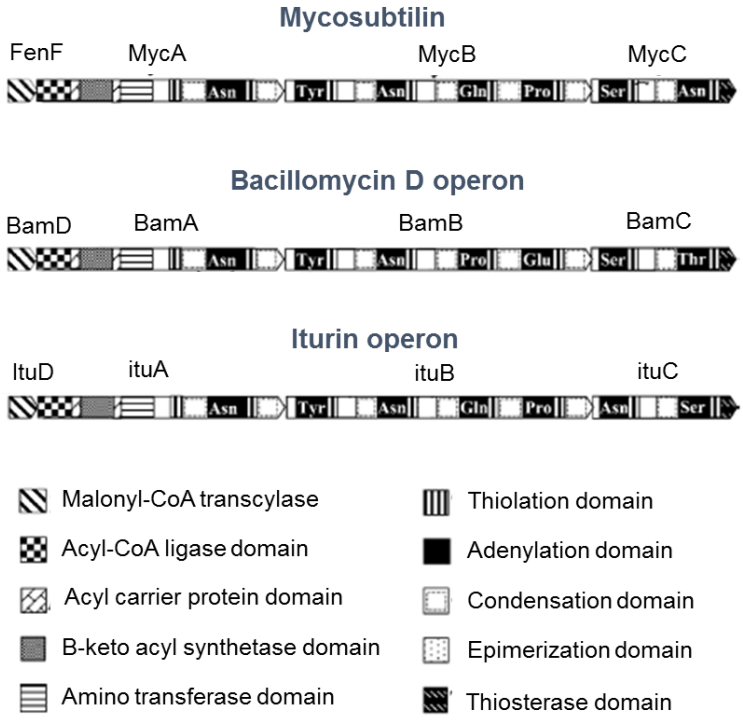


Figure II-1: Schematic diagram of mycosubtilin, bacillomycin D and iturin A operon from Moyne *et al*., 2004.

The mycosubtilin operon shares the same modular organisation as other peptide synthetase gene cluster, but additionally contains a fatty acid enzymatic domain along with a peptide enzymatic domain to form a hybrid synthetase in the second ORF mycA

(Leclère *et al.*, 2005). Another member of the iturin family, the iturin A synthetase operon share the same organisation as the mycosubtilin operon and spans a region more than 38 kb long, composed by four open reading frames: ituD, ituA, ituB and ituC (Tsuge *et al.*, 2001). The ituD gene encodes a putative malonyl coenzyme A transacylase, whose disruption results in a specific deficiency of iturin A production (Hsieh *et al.*, 2008).

In this study a characterisation, identification and evaluation of the antifungal potential of a set of bacterial isolates was undertaken with the assistance of molecular approaches.

Thus, this chapter focus on i) the selection of microorganism with antifungal potential, ii) detection of oligonucleotide regions responsible for the LPP antifungal compounds biosynthesis, and iii) identification of the bacterial strains with higher activity against biodeteriogenic fungi isolated from biodegraded cultural heritage artefacts.

3. Materials and Methods

3.1. Bacterial strains and culture media

For this study, 21 bacterial strains (Table II-2) isolated from artworks or from healthy *Quercus suber* were used. Microorganisms belonging to the culture collection of HERCULES Laboratory, Biotech laboratory, Évora University was maintained on nutrient agar slants and stored at 4ºC until use.

The bacilli strains selected as LPP producers were characterised by the morphological, physiological and biochemical characteristics based on the Bergey's manual of systematic bacteriology (Vos *et al.*, 2011) and by partial sequence of 16S rDNA.

3.2. Bioactive compounds production

For the production of potential bioactive compounds, the bacterial cells in a concentration of 2.975 x 10^9 CFU were inoculated in 100 mL of NB (Nutrient Broth) medium. The inoculum culture was incubated for 48 h at 30ºC in an orbital shaker at 150 rpm (IKA KS 4000 i control, Germany). The bacterial cells were removed from the culture broth by centrifugation (1 000 × *g* for 10 min at 4ºC). The supernatant was maintained at -20ºC for further analysis.

3.3. Antifungal activity assessment

Fungal spore suspensions of *Fusarium oxysporum, Aspergillus niger, Cladosporium* sp.*, Penicillium* sp.1*, Mucor* sp., *Rhodotorula* sp., *Penicillium* sp. 2, *Penicillium* sp.3 and *Alternaria* sp., were prepared by adding loopful of hyphae and spores from a MEA slant incubated at 25ºC for 7 days, to 5 mL of NaCl 0.85% solution. The suspension was filtered by sterilised cotton or triple gauze. A 10^6 CFU/mL spore suspension was obtained through dilution and the fungal suspensions were incorporated in MEA at 45ºC in Petri dishes. Filter paper discs (Macherey-Nagel 827 ATD) impregnated with 10 µL of *Bacillus* culture broth without cells, were placed on the agar and the Petri dishes were incubated at 25ºC for 24–48 h. Antifungal activity was indicated by the formation of inhibition zones around the discs where the *Bacillus* supernatant was applied (Caldeira *et al.*, 2008).

3.4. Liquid cultures growth conditions

Liquid cultures of the bacteria strains that have shown antifungal potential were performed in 100 mL of Nutrient Broth (HIMEDIA) medium. To monitor the microorganism growth, the cultures were incubated for 72 h at 30ºC in an orbital shaker

at 150 rpm (IKA KS 4000 I control) and the absorbance was periodically monitored at 600 nm.

The kinetics of the bacterial populations growth was fit by the Gompertz model that can be written as follows (Chowdhury *et al.*, 2007):

$$\mathrm{Ln\ N/N_0} = \mathrm{Ae^{(-e^{(-b-cx)})}}$$

where *N* is the decimal logarithm of microbial cells (Abs 600 nm) at time *t*; N_0 is the asymptotic log count as time decreases indefinitely; *A* is the number of log cycles of growth; *b* is the relative growth rate at time, t (h^{-1}) and *c* is the time required to reach the maximum growth rate (h).

The specific growth rate (μ) was determined from experimental data corresponding to the exponential phase of growth, using the relation,

$$\mu\ (h^{-1}) = \frac{(c \times A)}{e^1}$$

where μ is the specific growth rate of the microorganism and *e* is the *Neper* number. The generation time was calculated with the following expression (Zwietering *et al.*, 1990).

$$g(h) = \ln\frac{(2)}{\mu}$$

Assays were carried out in triplicate and non-linear regression modules of the software SigmaPlot (Version 12.0.0, Germany) was used to fit the data.

3.5. Bacterial DNA extraction

The cells of the *Bacillus* strains that have shown antifungal potential were collected from fresh slants and suspended in lysis buffer (40 mM EDTA, 400 mM NaCl, 50 mM Tris-hydrochloride, pH 9.0). These cells were disrupted by adding lysozyme to a final concentration of 1 mg/mL followed by incubation at 37°C for 20 min. After incubation, α-Chymotrypsin in 10% (w/v) Sodium Dodecyl Sulphate (SDS) aqueous solution was added to a final concentration of 4 mg/mL and SDS to a final concentration of 0.5% (w/v). The cell suspension was then incubated at 50°C for 30 min. DNA was

extracted by first adding a phenol/chloroform/isoamyl alcohol (25:24:1) volume equal to the aqueous phase, with a subsequent extraction of the aqueous phase using an equal volume of chloroform/isoamyl alcohol (24:1). DNA was precipitated overnight (-20°C) after the addition of absolute ethanol (2x aqueous volume) and 3 M sodium acetate (0.3x the volume of the aqueous phase). After this period DNA was collected by centrifugation the next day by centrifugation at 11 900 *g* for 25 min. DNA pellet was resuspended in sterile 1x TE buffer, pH 8 (Rinta-Kanto *et al.*, 2005). The extracted DNA was quantified using µDrop Plate (Thermo Scientific MultiScan Go, Wilmington, DE, USA), following the manufacturer's instructions. The system also provides the DNA absorption ratio 260/280 and 260/230. DNA extracts were diluted (1:5, 1:10 and 1:100) and stored at 4°C until analysis.

3.6. 16S ribosomal DNA sequence analysis

The genomic DNA of *Bacillus* strain extracted was used as a template. The 16S ribosomal DNA sequence was amplified and sequenced using universal primers 8F: 5'-AGAGTTTGATCATGGCTCAG-3' and 1492R: 5'-ACGGTTACCTTGTTACGACTT-3' (Zhao *et al.*, 2013). PCR reactions were carried out in a 25 µL reaction volume containing 1 µL genomic DNA, 2.5 µL 10× PCR buffer, 20 mM MgCl2, 0.2 mM of each dNTP, 0.1 µM of each primer and 1.25 U Taq DNA polymerase (Thermo scientific, Waltham, USA).

The amplification was conducted by polymerase chain reaction in a PCR thermal cycler (MJ Mini Bio-Rad; Laboratories Inc., USA). The PCR reactions were carried out as follow: an initial denaturing step at 95ºC for 5 min followed by 40 cycles at 94ºC each 1 min, 50ºC for 1 min, and 72ºC for 2 min. The reaction was completed by a 6 min extension at 72ºC. PCR products were analysed by agarose gel (1%) electrophoresis, stained by SYBR® Safe DNA 10 000x (Invitrogen, Waltham, USA), and sequenced by capillary electrophoresis using the ABI PRISM 3730 xl sequencer (Applied Biosystems) with the Kit BDT v1.1 (Applied Biosystems). The homology search of the determined 16S

rDNA sequences was carried out using the *Basic Local Alignment Search Tool - BLAST* 2.2.25+ from NCBI database (http://blast.ncbi.nlm.nih.gov) and Ribosomal Database Project (RDP-II) (https://rdp.cme.msu.edu/). The alignment of the homologous sequence was performed using BioEdit software (version 7.2.2.) (Hall, 1999).

Phylogenetic tree analysis were performed using MEGA 4.0 software (Tamura *et al.*, 2007) by the neighbor-joining analysis (Saitou and Nei, 1987).

3.7. Amplification of *Bacillus* lipopeptide genes

Genomic DNA was isolated from CCLBH 1051, CCLBH 1052, CCLBH 1053 and CCLBH 1054 strains as previously described. Primers used to amplify the genes encoding for bioactive compounds of *Bacillus* in test were either used as previously described by Joshi and Gardener (2006) and Cao *et al*. (2012) or designed using Primer Blast tool from NCBI company (Bethesda, USA), based on the consensus sequences of known *Bacillus* lipopeptide antibiotic genes deposited in GenBank (Table II-1) (Joshi and Gardener, 2006; Cao *et al.*, 2012).

Table II-1: Oligonucleotide primers used to detect genetic markers for biological activity in bacteria with antifungal potential.

Primer	Sequence 5'-3'	Target	Amplicon size (bp)
ITUCF1	TTCACTTTTGATCTGGCGAT	ituC	575
ITUCR3	CGTCCGGTACATTTTCAC	ituC	
bamB1F	AAGAAGGCGTTTTTCAAGCA	ituB	508
bamB1R	CGACATACAGTTCTCCCGGT	ituB	
ituD2F	GATGCGATCTCCTTGGATGT	ituD	647
ituD2R	ATCGTCATGTGCTGCTTGAG	ituD	
ituA1F	TGCCAGACAGTATGAGGCAG	ituA	885
ituA1R	CATGCCGTATCCACTGTGAC	ituA	

PCR reactions were carried out in a 25 µL reaction volume containing 1 µL genomic DNA, 2.5 µL 10× PCR buffer, 20 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer and 1.25 U Taq DNA polymerase (Thermo scientific, Waltham, USA). Amplification was performed with a PCR thermal cycler (MJ Mini Bio-Rad; CA, USA) programmed for one cycle of 94ºC for 5 min, followed by 35 cycles of 94ºC for 30 s, annealing for 30 s at 52ºC for ituC specific primer, whereas those for ituD, ituB and ituA were set to 58ºC and 72ºC for 1 min, and extension at 72ºC for 7 min was conducted after 35 cycles. A negative control without DNA was included in each PCR run and duplicates of each PCR reaction were performed. The amplified products were visualised by gel electrophoresis in a 1% agarose gel, stained by SYBR® Safe DNA 10 000x (Invitrogen, Waltham, USA).

3.8. Real-time PCR analysis

The reactions were carried out using the PerfeCTa SYBR® Green Supermix (Quanta BioSciences, Inc, Gaithersburg), containing SYBR® Green fluorophore, which fluoresces at 530 nm when bound to double-stranded DNA.

Each PCR reaction was performed in a total volume of 10 µL containing 9.6 µL of PerfeCTa® SYBR® Green Supermix, 0.05 µL of each primer (50 µM) and 0.4 µM of template bacterial DNA extracted. For each PCR assay two negative controls were included. For each sample, the DNA extract and its dilutions were analysed in duplicate.

The real-time PCR reactions were carried out in a PikoReal 96 (Thermo scientific, Waltham, USA) with a PikoReal™ Software 2.2, using a fast PCR protocol consisting of an initial denaturing step at 95°C for 7 min followed by 40 cycles, each consisting of 30 s of denaturation at 95°C, and 30 s of annealing-extension at 60°C. The fluorescence at 530 nm was monitored for real-time data collections during annealing-extension. Then, to construct the melting curve, 46 cycles of 10 s increasing the temperature in 0.5°C (from 72 to 95°C) were carried out, measuring the fluorescence at 530 nm after each of

them. Thermal cycling, fluorescent data collection and data analysis were carried out with a PikoReal™ Software, version 2.2 (Thermo scientific, Waltham, USA).

PCR line subtracted Cycle Fluorescence (CF) and Relative Fluorescence Units (RFU) was plotted and used to calculate the average background fluorescence emission in the initial cycles. Threshold fluorescence intensity was established at 10-fold higher than the standard deviation in the initial cycles. The PCR cycles at which fluorescence exceeded the threshold was defined as the Threshold Cycle (Ct). The absolute quantification was performed by comparing the Ct values of the unknown samples with the standard curve and carrying out appropriate calculations considering the initial amount of sample processed and the corresponding dilution. The concentrations of bacilli target genes are expressed as amount of specific DNA present in each sample (pg DNA/mg of strain).

3.9. LC-ESI-MS analysis

After bacterial cells removal, the supernatant obtained from the liquid culture centrifugation, 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-ESI-MS analyses were carried out in a LCQ Advantage ThermoFinnigan mass spectrometer equipped with an electrospray ionization (ESI) source and using an ion trap mass analyser. The conditions of the lipopeptides analysis were: capillary temperature 300ºC, 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 x 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 min^{-1}. The elution program followed a linear gradient from 20 to 50% of A (0–10 min) and from 50 to 100% of B (10–40 min).

4. Results and Discussion

4.1. Selection of bacterial strains with antifungal potential

The characterisation, identification and evaluation of the antifungal potential of a set of bacterial isolates was undertaken in order to establish the production of active lipopeptides against heritage biodeteriogenic fungi.

4.1.1. Screening of the antifungal activity

In order to select the bacterial strains producers of antifungal compounds, 21 bacteria isolated from artworks or from healthy *Quercus suber* were used for screening the antifungal activity. Batch cultures of these 21 selected bacteria were performed and after 48 h of incubation, the cells were removed and the supernatants of the cultures were used on antifungal assays (Table II-2).

The results showed that from the bacterial strains tested, four of these strains reveal great antifungal activity against fungi isolated from biodegraded historic materials. In further assays, these strains will be used in order to explore the antagonistic potential to produce new biocides for cultural heritage rehabilitation.

Table II-2: Antifungal activity assay with 21 bacteria strains isolated against biodeteriogenic fungi.

***Bacillus* sp.**	**Biodeteriogenic Fungi**								
	Fusarium oxysporum CCMI 898	*Aspergillus niger* CCMI 296	*Penicillium* sp.1	*Cladosporium* sp.	*Mucor* sp.	*Rhodotorula* sp.	*Penicillium* sp.2	*Penicillium* sp.3	*Alternaria* sp.
CCLBH 1051	++	+++	+++	++	+++	+	++	+++	+++
CCLBH 1052	++	+	+	++	++	+	+	++	++
CCLBH 1053	+++	+++	+++	+++	+++	++	++	+++	+++
CCLBH 1054	+	++	+	-	+	-	+	+	-
IL13	+/-	-	-	+/-	+/-	-	-	+/-	-
Z2B	-	-	-	-	+/-	-	-	+/-	-
Z2C	+/-	-	-	+/-	-	-	+/-	-	-
P2-2-A1	-	+/-	-	-	+/-	-	-	-	+/-
P2B1	-	-	+/-	+/-	-	-	-	-	-
P4-6-brown	+/-	-	-	-	+/-	-	+/-	-	-
B38	-	-	-	-	-	-	-	+/-	-
Z2E	-	-	-	+/-	-	-	-	-	+/-
P3-A1-B1	-	+/-	-	-	+/-	-	-	+/-	-
IL10a	-	-	-	-	-	-	-	-	-
FS19a	+/-	-	-	-	+/-	-	-	-	+/-
Z4B	-	-	-	-	-	-	-	-	-
P2B5	-	+/-	-	-	-	-	-	-	-
B31	-	-	-	+/-	+/-	-	-	+/-	+/-
B39	+/-	-	+/-	-	-	-	-	-	-
Z1A	-	+/-	-	-	-	-	-	-	-
Z2A	-	-	+/-	-	+/-	-	+/-	-	-

- Without inhibition; +/- not determined; + positive test (inhibition halo <15mm); ++ (inhibition halo 15–20mm); +++ (inhibition halo >20 mm).

The results of the inhibition assays allowed to verify that CCLBH 1051, CCLBH 1052, CCLBH 1053 and CCLBH 1054 strains have inhibitory capacity against all biodeteriogenic fungi tested, however depending on the biodeteriogenic target fungal strain, the bacteria present different levels of antifungal capacity. In fact, this strains have the capability to produce bioactive compounds that were released to liquid culture medium.

4.1.2. Amplification of *Bacillus* lipopeptide genes

Some strains of *Bacillus* are known for the ability to produce a variety of lipopeptides with remarkable surface and antimicrobial activities especially the nonribosomally synthesised cyclic lipopeptides surfactin, iturin and fengycin (Caldeira *et al.*, 2008; Rückert *et al.*, 2011; Zhao *et al.*, 2013). Previous works reported that the bacteria strains studied in this work have a great potential to produce lipopeptides from the fengycin and specially iturin family (Caldeira *et al.*, 2008; Caldeira *et al.*, 2011b).

In order to confirm and identify DNA markers on genome of these strains, corresponding to NRPS, Polymerase Chain Reactions (PCR) were conducted to detect genes involved in the biosynthesis of iturin family lipopeptides reported to be produced by the *Bacillus* strains.

A total of 4 gene fragments were efficiently amplified by ituD2F/ituD2R, ituA1F/ituA1R, bamB1F/bamB1R and ITUCF1/ITUCR3 primers (Joshi and Gardener, 2006). Primers used to amplify the genes encoding for bioactive compounds were either used for the amplification of the iturin A operon which spans a region more than 38 kb long and is composed of four Open Reading Frames (ORF's): ituD, ituA, ituB, and ituC.

The PCR products present in the agarose gel electrophoresis (Figure II-2), confirmed the integrity and the identity of these genes. It is possible to observe fragments with 885 bp, 508 bp, 575 bp and 647 bp corresponding to the four ORF's ituA, ituB, ituC and ituD, respectively.

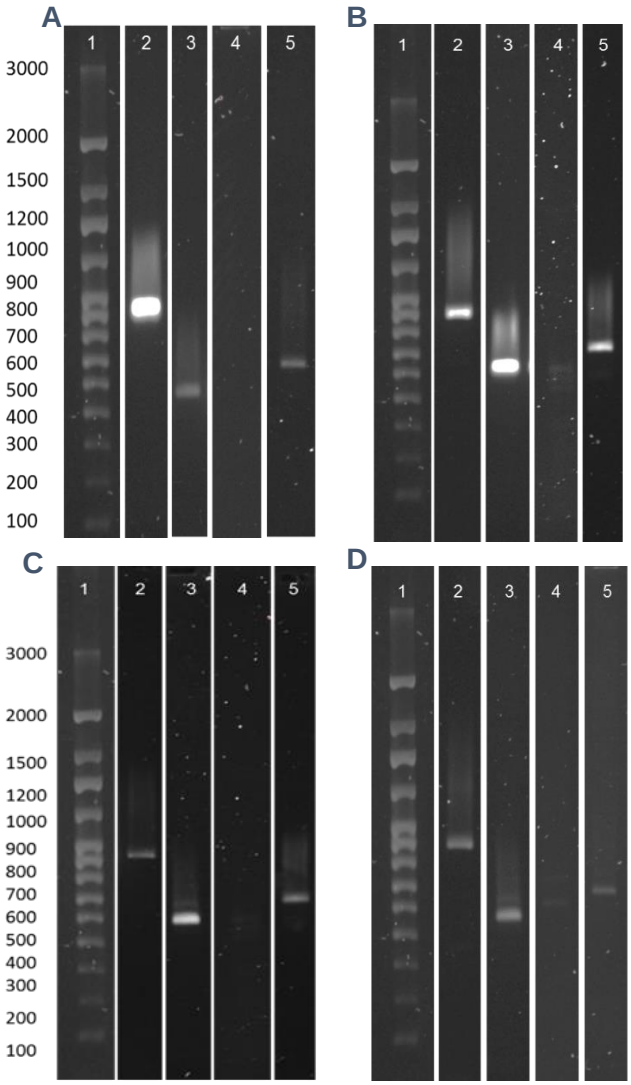


Figure II-2: Agarose gel electrophoresis of DNA isolated from different selected strains. A- CCLBH 1051; B- CCLBH 1052; C- CCLBH 1053; D- CCLBH 1054; Lane 1- 100 bp Ladder (Nzytech Ladder VII); Lane 2- PCR product of bacterial amplification with ituA primer; Lane 3- PCR product of bacterial amplification with ituB primer; Lane 4- PCR product of bacterial amplification with ituC primer; Lane 5- PCR product of bacterial amplification with ituD primer.

The PCR products obtained from the total DNA of the selected strains with different sizes showed several distinguishable bands with different levels of intensities. All the primers used in this study amplified fragment regions of iturin synthetase or its closely related compounds in the iturin family.

The primers corresponding to the ituA (ituA1F/ituA1R), ituB (bamB1F/bamB1R) and ituD (ituC2F/ituC2R) were well represented in the agarose gel electrophoresis with high intensity bands, in contrast with ituC (ITUCF1/ITUCR3) which were not present in the strains CCLBH 1051 and CCLBH 1053 electrophoretic gel.

Sequencing strategies of the obtained PCR products could be applied for the variability study of the nonribosomal protein synthases responsible for the iturinic compounds biosynthesis in the genome of the selected species, such as Next Generation Sequencing which will allow the full sequencing of the microbial population and the study of the genetic variability.

4.2. Quantification of iturin genetic expression

Real-time PCR was carried out to determine the copy number of the target DNA present in the bacteria strains genome. The DNA copy number varied from 6.5 $\times 10^3$ to 1.4 $\times 10^6$ copy/µg DNA in 1 mL of bacterial liquid culture, for the iturinic ORF's corresponding primers (Figure II-3).

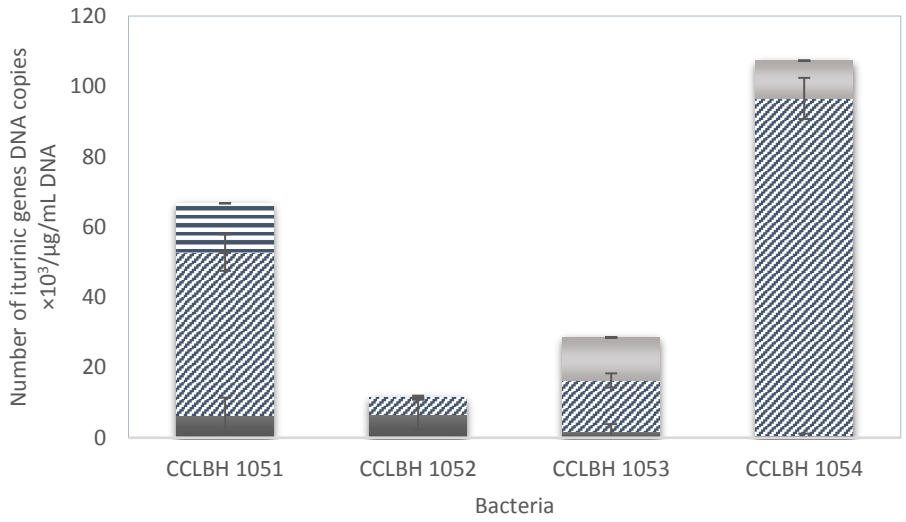


Figure II-3: Iturinic ORF's amplified DNA copies in the genome of the selected bacteria strain determined with real time PCR. A 10-fold serial dilution of pDNA CCLBH 1053 strain (3.4 × 10^3 to 3.4 × 10^8 copies/µL) was used to construct the standard curve for which two primer (■ itu A; ▨ itu B; ■ ituC; 〓 ituD).

The high number of iturinic ORF's amplified DNA copies was obtained in CCLBH 1054 strain analysis with the ituB corresponding primers. In all the bacteria strains the expression of ituB was well represented in contrast with the rest of the ORF's whose it expression differed according with the amplified strain.

The bioactive compounds iturin A, bacillomycin D as well as mycosubtilin, are members of the iturin group and has already been full sequenced (Tsuge *et al.*, 2001; Yao *et al.*, 2003; Moyne *et al.*, 2004; Zhao *et al.*, 2013). Several studies described that the operon of bacillomycin D is 98% identical to iturin A and 79% to mycosubtilin. Despite the similarity, the difference observed in the expression and gene quantification analysis of the iturin genes of the selected strains and between the iturin A operon, respectively, can be due to possible nucleotide differences in the multifunctional peptide synthetases responsible for the iturin A synthesis.

Therefore, the DNA sequences obtained from these amplifications confirmed the presence and different quantity of the iturin genes in the different strains. This evidenced that the approach used here could be considered in the future as an initial methodology for identification and selection of lipopeptides producer's strains. In fact, the presence, variability and expression of the nonribosomal protein synthases responsible for the iturinic compounds biosynthesis allows the strains selection with higher potential for fungal proliferation control. These selected microorganisms will be used to produce natural green safe biocides for cultural heritage artworks safeguard.

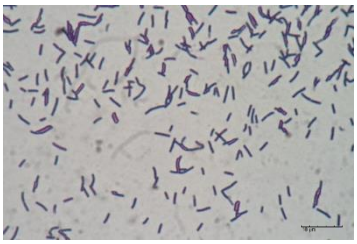
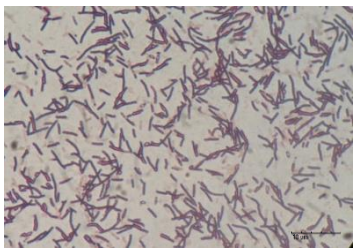
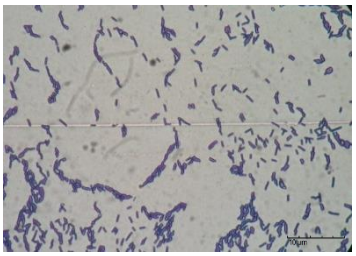
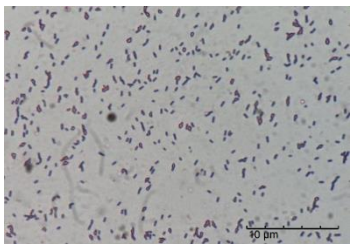
4.3. Identification and characterisation of bacterial strains with antifungal potential

Several approaches generally based on conventional phenotypic methods, encompassing culture and growth patterns on specific media, Gram staining, and morphological and biochemical characteristics have been proposed to classify *Bacillus* sp. (Xu and Cote, 2003; van Veen *et al.*, 2010; Vardhan *et al.*, 2011).

The characterisation of the selected bacteria was performed according to macroscopic (colour, size and morphology) and microscopic features (colour of colonies) and by DNA sequencing. The bacterial strains with a higher level of antagonist potential are Gram-positive spore-forming Bacilli, with aerobically growth.

The Table II-3 shows the macroscopic, microscopic and morphologic features of the bacterial strains in analysis.

Table II-3: Macroscopic and microscopic features of the selected microorganisms in analysis.

Identification	Macroscopic features of colonies	Microscopic Features (1000x)	Morphology
CCLBH 1051			Bacilli (Gram-positive)
CCLBH 1052			Bacilli (Gram-positive)
CCLBH 1053			Bacilli (Gram-positive)
CCLBH 1054			Bacilli (Gram-positive)

The results (Table II-3) allowed to observe the morphological characteristics of the bacterial strains with antifungal properties, against the tested biodeteriogenic fungi as was shown in previous results (Table II-2). These strain had the same morphological and biochemical characteristics, possibly belonging to the genera *Bacillus.*

Partial 16S rDNA gene sequences have been used for identification and classification of bacterial species and related genera (Kim *et al.*, 2010b). A comparative

analysis of 16S rDNA gene sequences of different species can reveal different phylogenetic cluster.

In the present study, we aimed to identify the bacterial strains and to determine whether or not a part of the 16S hypervariable sequence could be informative enough to relate the selected strains and to find possible phylogenetic association within other strains with antifungal potential.

The CCLBH 1051, CCLBH 1052, CCLBH 1053 and CCLBH 1054 16S ribosomal DNA sequence was amplified and sequenced using the universal primers 8F and 1492R (Zhao *et al.*, 2013). The amplification was conducted by PCR and fragment sequences of 1,5 kbp were obtained after agarose gel electrophoresis (Annexe C). After the determination of 16S ribosomal DNA concentration (Annexe D), sequencing and homology search in the Ribosomal database (RDP) were done. The results of the alignments revealed the homology within the 16S region.

The Table II-4 to Table II-7 show the seven more similar microorganism and the gene bank accession number of the CCLBH 1051, CCLBH 1052, CCLBH 1053 and CCLBH 1054 strains.

Table II-4: Homology match from the Ribosomal database (RDP) of the CCLBH 1051 16S rDNA.

Bacteria strain	Gene bank Acession	Reference
***Bacillus subtilis* 407D3**	HM099655	(Du and Lin, 2010)
***Bacillus subtilis* NBPP61**	FJ973542	(Yadav *et al.*, 2011)
***Bacillus amyloliquefaciens* EA1-10**	JF496398	(Liu *et al.*, 2011b)
***Bacillus amyloliquefaciens* H102**	HQ407277	(Gupta *et al.*, 2012)
***Bacillus subtilis* MO4**	AY553097	(Caton *et al.*, 2004)
***Bacillus* sp. TKSP21**	AB017591	(Lee *et al.*, 2003)
***Bacillus subtilis* B-FS01**	DQ520955	(Hu *et al.*, 2007)

Table II-5: Homology match from the Ribosomal database (RDP) of the CCLBH 1052 16S rDNA.

Bacteria strain	Gene bank acession	Reference
***Bacillus subtilis* F3-7**	EU882849	(Zhang *et al.*, 2008a)
***Bacillus* sp. G1**	EU257695	(Zhao *et al.*, 2007)
***Bacillus subtilis* F1-5**	FJ392725	(Zhang *et al.*, 2008b)
***Bacillus amyloliquefaciens* LD5**	GQ853414	(Huang and Li, 2009)
***Bacillus amyloliquefaciens* HS8**	GU323369	(Zhu and Xie, 2010)
***Bacillus amyloliquefaciens* GD4a**	HM055603	(Wen, 2010)
***Bacillus* sp. DYJL4**	HQ317147	(Xu *et al.*, 2010)

Table II-6: Homology match from the Ribosomal database (RDP) of the CCLBH 1053 16S rDNA.

Bacteria strain	Gene bank acession	Reference
***Bacillus amyloliquefaciens* zzx18**	KJ009413	(Zhang and Mo, 2014)
***Bacillus methylotrophicus* LK6**	KC790234	(Li *et al.*, 2014)
***Bacillus vallismortis* ZZB07**	JQ765432	(Chen, 2012)
***Bacillus amyloliquefaciens* ML471**	KC692205	(Sinacori, 2013a)
***Bacillus subtilis* X-272**	HQ262534	(Liu *et al.*, 2011a)
***Bacillus* sp. Q2-S3**	JX994137	(Peng *et al.*, 2013)
***Bacillus amyloliquefaciens* ML361**	KC692163	(Sinacori, 2013b)

Table II-7: Homology match the Ribosomal database (RDP) of the CCLBH 1054 16S rDNA.

Bacteria strain	Gene bank acession	Reference
***Bacillus* sp. FPZSP072**	JX867956	(Ramos, 2012)
***Bacillus pumilus* VKK-4NL**	JX852571	(Kalia *et al.*, 2012)
***Bacillus safensis* 0312MAR27A3**	LN774756	(Dominguez Monino, 2015)
***Bacillus* sp. 14076**	JN874793	(Liu, 2012)
***Bacillus pumilus* ZK1**	JQ773350	(Kee *et al.*, 2012)
***Bacillus pumilus* WS31**	JN210909	(Zhang *et al.*, 2012)
***Bacillus pumilus* ML477**	KC692169	(Sinacori, 2013b)

The sequence similarity research from the Ribosomal database (RDP) for the selected strains under study showed that CCLBH 1051 present higher similar score with two strains of *Bacillus subtillis* (*Bacillus subtilis* 407D3 and *Bacillus subtilis* NBPP61) followed by two *Bacillus amyloliquefaciens* strains. The homology of CCLBH 1052 strain indicated a particular low similarity score with *Bacillus subtilis* F3-7, which may indicate that this strain can perhaps to be a new specie within the *Bacillus* genera and with characteristics related to *Bacillus subtilis* and *Bacillus amyloliquefaciens*. In fact, these two species are phenotypically similar and can be easily mistaken (Reva *et al.*, 2004).

Analysis of the multiple alignment (Annexe E), ranging from 56 bp to 620 bp, revealed a variable nucleotide at site 264, where the CCLBH 1051 strain and *Bacillus subtillis* 407D3 sequences showed a guanine instead of an adenine residue observed in the remaining sequences. Also, in the 612 position only CCLBH 1052 strains showed a guanine differentiating from the others sequence that present a cytosine.

Reva *et al*. (2004) revealed that T/A pair at positions 185/202 was present in most of the sequenced strains but it was altered to C/G in the plant-associated strains of *B. amyloliquefaciens* (Reva et al., 2004). In this case, the alignment results showed two tyrosine in these positions, evidencing some level of differentiation from the species from the previous study.

For the CCLBH 1053 strain the Ribosomal database (RDP) suggested a similarity score of 0.929 with *Bacillus amyloliquefaciens* zzx18 and 0.928 with *Bacillus methylotrophicus* LK6 and *Bacillus vallismortis* ZZB07. These three *Bacillus* strains in previous studies revealed a clear phylogenetic affinity with pairwise similarities ranging from 98.2 to 99.2% (Madhaiyan *et al.*, 2010) making the discrimination within the species difficult to achieve.

Finally, the research for CCLBH 1054 strain revealed a maximum similarity score for *Bacillus* sp. FPZSP072, *Bacillus pumilus* VKK-4NL and a lower score with *Bacillus safensis.* In fact, these two strains share some phenotypic and biochemical

characteristics on 16S rDNA gene sequences which led authors to research possible ways to discriminate the two strains (Branquinho *et al.*, 2014).

Additionally, the results of the multiple alignment between CCLBH 1054 strain and *Bacillus pumilus* VKK-4NL (Annexe E), show some level of differentiation from the remaining strains in analysis (specially *Bacillus subtillis* and *Bacillus amyloliquefaciens)*, suggesting a phylogenetic distance from the previous strains and a high level of similarity between each other.

The identification of *Bacillus* species within the genera is challenging. Some of the *Bacillus* strains almost share identical 16S rDNA gene sequences (99.2 – 99.6%) which usually illustrate limited diversity between members of closely related strains (Mohkam *et al.*, 2016).

There is no consensus in what defines a species and genus based on the 16S rDNA sequence similarity. However, similarity of $\geq$ 99% for 16S sequence, when compared with the prototype strain sequence in GenBank, can be considered as a criteria for the classification of species. Also, for the identification at the genus level, the criteria usually used can be defined as a 16S rDNA sequence similarity of $\geq$ 97% (Drancourt *et al.*, 2000; Xu and Cote, 2003; Vardhan *et al.*, 2011; Mohkam *et al.*, 2016).

Studies reported by other authors shows that some of the most similar *Bacillus* strains (Table II-4 to Table II-7) researched have shown antagonistic capacity against filamentous fungi. *Bacillus subtilis* MO4 produces antifungal metabolites that caused abnormal swelling of hyphae (Yang *et al.*, 2009). *Bacillus subtilis* strain B-FS01, isolated from rape (*Brassica napus*), exhibits predominantly antagonistic activities against *Fusarium moniliforme* Sheldon ATCC 38932, revealing the presence of fengycin homologues in HPLC analysis (Hu *et al.*, 2007).

Additionally Li *et al*., 2014 showed that *Bacillus methylotrophicus* LK6 presents biocontrol potential against soilborne pathogens (Li *et al.*, 2014). Zhang *et al*., 2012 in

addition to sequence *Bacillus pumilus* WS31, reported also that this strain could be used as an effective tool for isolating plant growth-promoting rhizobacteria strain.

4.4. Molecular phylogeny analysis

The analysis of amplification of 16S rDNA were also used to determine the *Bacillus* phylogeny within the genera and the possible relations between the strains.

16S rDNA and 1,5 Kbp fragment sequence were analysed and their relationships were established by constructing the corresponding dendogram and phylogenetic tree with neighbor-joining. The phylogenetic tree was constructed using bootstrapping analysis (Figure II-4).

Phylogenetic analysis on the basis of the sequences of 16S rDNA were performed, showing that the strains form a monophyletic clad, which was closest to but could be clearly distinguished from *B. subtillis*, *B. amyloliquefaciens, B. safensis* and *B. pumilus*. Molecular and phenotypic characterisation showed that the *Bacillus* CCLBH 1052 might belong to a new subspecies of *Bacillus* sp..

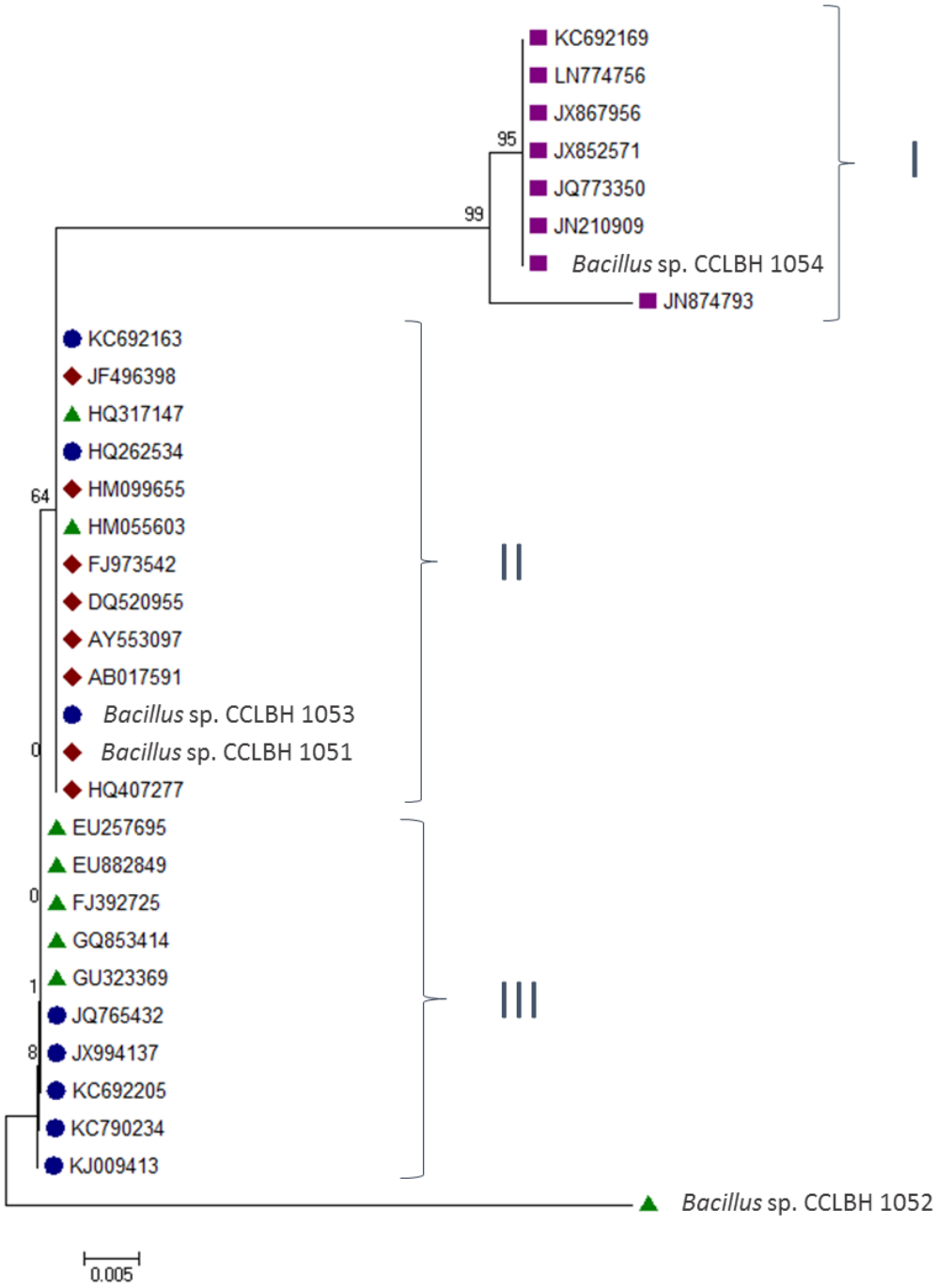


Figure II-4: Phylogenetic relationships of *Bacillus* species based on nucleotide sequences of the 16S rDNA. The tree was generated by the neighbor-joining method (MEGA 4.0 software). Percentages at nodes represent levels of bootstrap support from 1000 resampled datasets. The bar indicates 0.005% estimated sequence divergence.

The major clusters (Figure II-4) were conserved as it can be seen by comparing clusters I, II and III, for instance *B. safensis* and *B. pumilus* are found in cluster I of the

phylogenetic trees similarly members of cluster II and III (*B. subtilis* and *B. amyloliquefaciens).* The *Bacillus* sp. CCLBH 1051 and *Bacillus* sp. CCLBH 1053 strains belongs to the same group (cluster II) where are also included several strains of *B. subtilis* and *B. amyloliquefaciens,* presenting a high level of phylogenetic relationship with these strains. In the cluster I, the *Bacillus* sp. CCLBH 1054 exhibits a separation of the remaining groups, showing a high level of approximation with sequenced *B. pumilus* strains. Only *Bacillus* sp. CCLBH 1052 shows a low level of phylogenetic relation in comparison with all the strains analysed, presenting a major genetic deviation with *B. subtilis* and *B. amyloliquefaciens,* also confirmed by the homology results within the 16S region.

4.5. Bacterial growth profile

Batch cultures were performed as described on the Materials and Methods section and the time course profile of the four strains of *Bacillus* sp. and their specific growth rate was determined. The results are shown in Figure II-5.

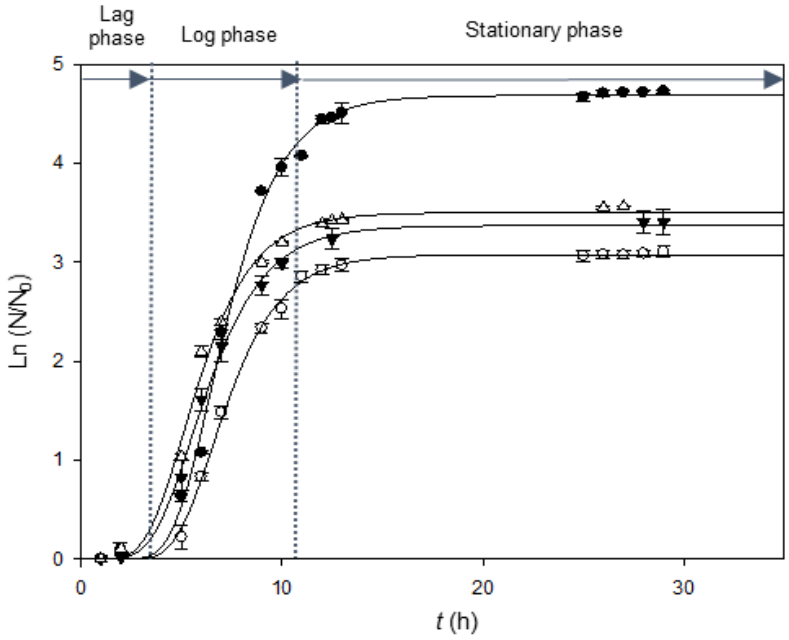


Figure II-5: Time course profiles of *Bacillus* sp.. All data was determined in triplicate. *Bacillus* CCLBH sp. 1051 (–●–); *Bacillus* sp. CCLBH 1052 (–o–); *Bacillus* sp. CCLBH 1053 (–▼–); *Bacillus* CCLBH sp. 1054 (–Δ–).

All cultures present similar kinetic features, namely a lag phase during about 3 h, a period of exponential growth of approximately 8-11 h and a stationary phase, after 12 h of culture.

Table II-8: Specific growth rate and generation time values for the *Bacillus* sp. strains selected.

***Bacillus* sp.**	**Specific Growth Rate μ (h^{-1})**	**Generation Time g (h)**
CCLBH 1051	0.89 ± 0.10	0.78 ± 0.19
CCLBH 1052	0.59 ± 0.06	1.17 ± 0.02
CCLBH 1053	0.60 ± 0.06	1.15 ± 0.10
CCLBH 1054	0.69 ± 0.10	1.00 ± 0.04

The specific growth rate and the generation time was determined from the exponential growth period (Table II-8) of the four strains tested. The *Bacillus* sp. CCLBH 1051 strain displayed the higher specific growth rate (μ) and the lower generation time (g).

The bioactive compounds were recovered on stationary phase when nutrients starvation limit their growth. Starvation can be achieved 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.

In order to proceed with LPP production confirmation, a LC-ESI-MS analysis of the supernatant of cultures was performed. As example, culture of *Bacillus* sp. CCLBH 1051 is presented (one of the most representative producer strains). The chromatogram showed several molecules around peaks at m/z 1031 and 1045 (Figure II-6).

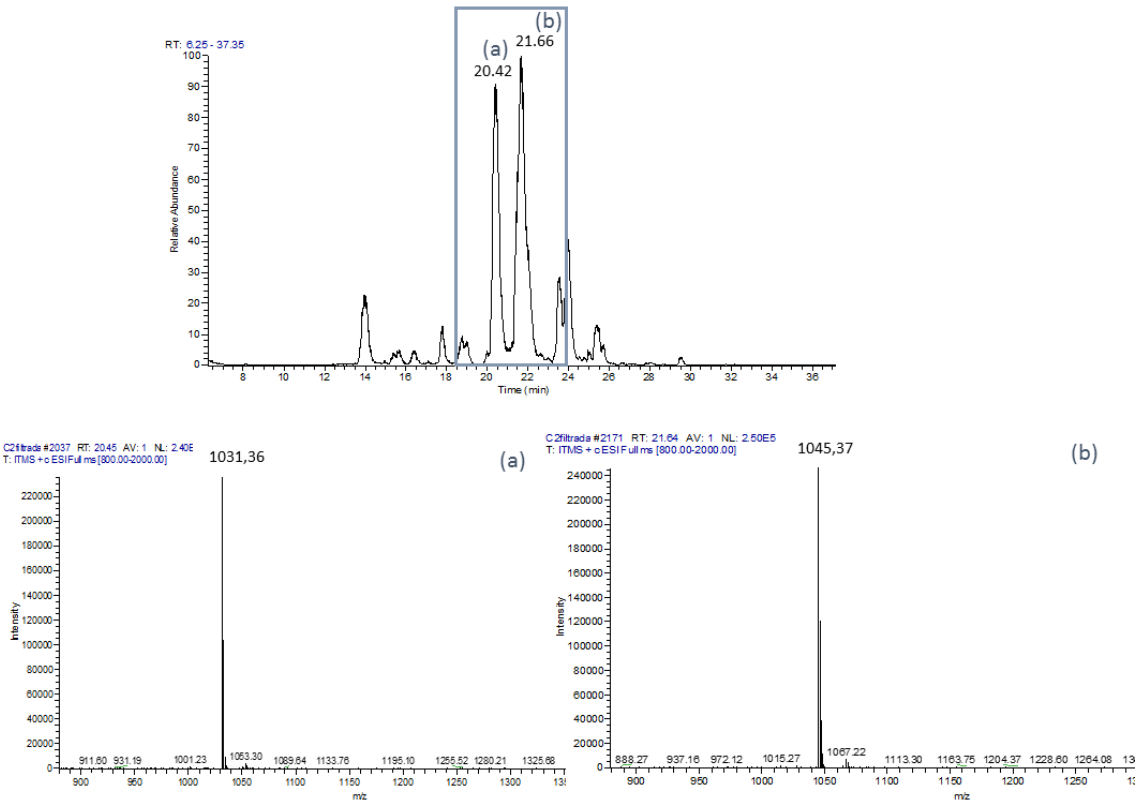


Figure II-6: Total ion chromatogram of the supernatant liquid culture of *Bacillus* sp. CCLBH 1051 and mass spectra corresponding to the peaks (a) m/z 1031.36 and (b) m/z 1045.37.

The mass spectra obtained in a ESI mode, showed a cluster containing molecules with a difference of 14 Da, corresponding to the loss of CH_2 suggesting a series of homologous molecules with different length of fatty acid chain. This m/z values suggest the presence of the antifungal lipopeptide mainly from iturin family. These results will be analysed with more detail in Chapter IV.

This combined methodology with antifungal screening and molecular data constitutes a valuable tool for quick identification of iturin-producing strains, constituting an effective approach for confirming the selection of lipopeptides producer strains. Therefore, the ability of *Bacillus* sp. CCLBH 1051, CCLBH 1052, CCLBH 1053 and CCLBH 1054 to produce antifungal compounds with antagonistic activity against biodeteriogenic fungi has been well stablished in this work. Thus, these bacterial strains

will be used to developed new mitigation strategies namely to produce natural green safe biocides for cultural heritage goods safeguard.

5. Conclusions

Twenty-one bacterial strains available on the culture collection of HERCULES Laboratory (Biotech laboratory) were study in order to access the promising strains with biocontrol potential. In fact only four, in a total of 21 strains, have higher ability to suppress fungal growth with different levels of efficiency. These bacterial strains exhibit the same morphological and biochemical characteristics, belonging to the genera *Bacillus*. The four strains show the same growth profile.

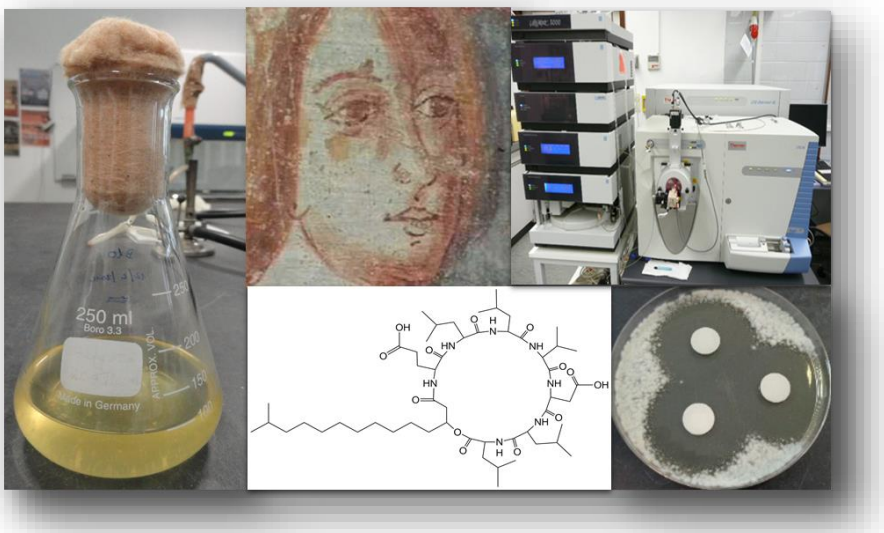
The 16S rDNA of the selected strains shows a high level of similarity with sequenced strains of *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Bacillus pumilus*.

For the establishment and quantification of the gene fragments associated to antibiotic biosynthesis a Real time-PCR assay was conducted. The gene fragments associated with the ORF's involved in antibiotic synthesis (ituA, ituC, ituB and ituD) were efficiently amplified and quantified. Therefore, the DNA sequences obtained from these amplifications confirmed the presence and different expression of the iturin genes, constituting an effective approach for the identification and selection of lipopeptides producer strains. The developed PCR and Real-time PCR method could be considered a potential tool for quick identification of iturin A-producing strains.

The presence, variability and expression of the nonribosomal protein synthases responsible for the iturinic compounds biosynthesis allows to select the strains with higher potential for biocontrol to be used as a natural green safe biocides for biodegraded cultural heritage artefacts.

CHAPTER III

Bioactive compounds detection: antifungal potential in cultural heritage context



Some results of this chapter were published in the following scientific publications:

Silva M., Silva S., Teixeira D., Candeias A., Caldeira A.T. (2014). Production of novel biocides for cultural heritage from *Bacillus* sp. *in* Science, Technology and Cultural Heritage. Rogerio-Candelera (Ed), CRC Press/Balkema Taylor and Francis Group, London, UK, pp. 223-229 ISBN: 978-1-138-027-44-2.

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 6 (10): 788-796

1. Overview

The growth control of microflora in cultural and built heritage is usually performed by treatments with toxic chemicals. It is crucial for the safeguard of our precious heritage, the development of strategies to uncover new green safe molecules. The use of these compounds allow to apply effective approaches against the biodeteriogenic agents responsible for cultural and built heritage degradation without detriment of the environment.

Therefore, the main goal of this chapter was to develop methodological basis for bioactive compounds detection in order to carry out proper remediation strategies, based on green biocides treatment, to promote historic materials longevity.

A methodology based on the combination of antifungal tests with spectroscopic (NMR and FTIR-ATR) and chromatographic techniques (LC–ESI-MS) was implemented. This combined approach, without need of the laborious previous isolation, allowed to detect and access a chemical characterisation of these ground-breaking bioactive compounds and, simultaneously, a fast screening and evaluation of their antifungal potential.

These methods can be employed for optimisation and large-scale production of the new biocides from *Bacillus* cultures which can be used as viable alternative to toxic chemical biocide, normally applied in cultural and built heritage rehabilitation.

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2. Introduction

Biological colonisation through 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 the degradation/deterioration of these materials. Microorganisms, such as bacteria, fungi, algae and lichens, are key-players (Rojas *et al.*, 2009) in the damage of materials with historic value and the action of specific enzymes and organic acids, produced by microbial communities, plays a determinant role (Urzì and De Leo, 2007).

However, filamentous fungi are particularly dangerous because their hyphae may have high level of proliferation and their spores, in a dormant state, are commonly present and available for germination. Also 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 (Tran *et al.*, 2012; Rosado *et al.*, 2014).Thus, the proliferation of fungi in heritage artefacts can lead the appearance of stains, inducing discoloration and deterioration of their surface, and the detachment of fragments (Milanesi *et al.*, 2006; Guiamet *et al.*, 2011).

In this way, carrying out proper remediation strategies for microbiologically contaminated historic materials is of vital importance to ensure its preservation.

Biocide treatments are considered to be one of the practical approaches for bioremediation and conservation of artworks (Urzì and De Leo, 2007). Nevertheless, protective solutions based on the use of toxic chemical compounds that can be accumulated in animal tissues (Fonseca *et al.*, 2010) face increasing restrictions. In this way, innovative research is needed to replace these biocides in conservation and restoration of artworks by green solutions that do not present negative effects on the environment or human beings (Ashraf *et al.*, 2014).

Bacillus species can be used as a natural source for the production of these green biocides. They synthesise a great diversity of secondary metabolites with biological activity (Mikkola *et al.*, 2004), namely antagonistic activities against several pathogenic fungi. Some strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been referred to produce antifungal peptides (Tsuge *et al.*, 2001; Caldeira *et al.*, 2007; Ben Slimene *et al.*, 2012). The lipopeptides surfactin, fengycin and iturin are amphiphilic membrane-active biosurfactants with potent antifungal activities (Moyne *et al.*, 2004) which can be used as additive in new harmful and environmental friendly biocides.

These lipopeptides share similar amphipathic structures containing both a hydrophilic peptide portion and a hydrophobic fatty acid portion. Most of the lipopeptides have a cyclic structure mediated by the linkage between a C-terminal peptide residue and a β-hydroxy fatty acid, hydroxyl group of the peptide residue, or β-amino acid.

NRPSs are multi-modular enzymes that recognise, activate, modify and link the amino acid intermediates to the product peptide (Roongsawang *et al.*, 2010). They are capable of synthesising peptides that contain unusual amino acids including D-amino acids, α-amino acids, and hydroxy- or N-methylated amino acids (Varadavenkatesan and Murty, 2013).

Bacillus subtilis is one of the most versatile producers of cyclic lipopeptides, such as surfactin, iturin and fengycin (Garbay-Jaureguiberry *et al.*, 1978; Cho *et al.*, 2003; Stein, 2005; Cao *et al.*, 2009).

The lipopeptide of the iturin family consists of iturins A-E, mycosubtilin and bacillomycin D, F and L. These amphiphilic cyclic peptides contain seven α-amino acids (A1-A7) and one unique β-amino acid in their composition. Iturin A, for example, in nature is produced as a mixture of up to eight isomers that are iturins A1-A8 (Ruangwong *et al.*, 2012a). The iturin group – bacillomycines, bacillopeptines and mycosubtilines comprises lactams containing a C_{14}-C_{17} 3-amino-fatty acid and amino acids, the 2nd and 3rd one being always $_D$-Tyr-$_D$-Asn (Schneider *et al.*, 1999).

In the case of surfactins, they are classified in three different types (A, B and C)

according to their amino acid sequences. *Bacillus subtilis* strains do not produce a unique type of surfactin, but rather a natural diversity of homologues, which differ in the length and ramification of the fatty acid chains, and isoforms, characterised by some differences in the peptidic sequence (Pereira *et al.*, 2013). These LPP are constituted by lactones incorporating a C_{13}-C_{15} 3-hydroxy fatty acid and seven amino acids two of which are variable ($_L$-Glu-$_L$-Leu-$_D$-Leu-$_L$-X^4-$_L$-Asp-$_D$-Leu-$_L$-X^7) (Kim *et al.*, 2004).

In contrast to the surfactins where the ester bond is formed between the C-terminal amino acid and the hydroxyl group of the fatty acid, in the fengycins it connects the C-terminal amino acid and Tyr in the sequence. Two types of LPP fengycins (fengycin A and B), based on their amino acid sequences, has been described. Authors suggested for these LPP a lactonic structure consisting of a C_{16}-C_{18} 3-hydroxy-fatty acid followed by $_L$-Glu-$_D$-Orn-($_D$ or $_L$)-Tyr-$_D$-aThr-$_L$-Glu-$_D$-(Ala or Val)-$_L$-Pro-$_L$-Glu-($_L$ or $_D$)-Tyr-$_L$-Ileu (Stein, 2005; Ongena and Jacques, 2008). For each homologue, if the amino acid at position 6 is $_D$-Ala, it is classified as fengycin A, and if the amino acid is $_D$-Val for the same position, it is named by fengycin B (Wang *et al.*, 2004).

Therefore, the main goal of this chapter was to develop methodological basis for bioactive compounds detection and characterisation, without previous total isolation, in order to carry out proper remediation strategies to promote historic materials longevity, based on the use of green biocides.

3. Materials and Methods

3.1. Microorganisms maintenance

The strains of *Bacillus* sp. CCLBH 1051, CCLBH 1052 and CCLBH 1053 (Caldeira *et al.*, 2008) were maintained on NA (Nutrient Agar) slants and stored at 4ºC. Strains of *Fusarium oxysporum*, *Aspergillus niger*, *Cladosporium* sp., *Penicillium* sp.1, *Mucor* sp. *Rhodotorula* sp, *Penicillium* sp. 2, *Penicillium* sp.3 and *Alternaria* sp. were

isolated from biodegraded mural paintings. The fungal cultures, used as test microorganisms, were maintained on MEA (Malt Extract Agar) slants and also stored at 4ºC. The fungal and bacteria cultures belong to the laboratory collection (HERCULES-Biotech laboratory, Évora University)

3.2. Growth conditions and bioactive compounds production

For the production of bioactive compounds, the *Bacillus* sp. CCLBH 1051, CCLBH 1052 and CCLBH 1053 cells (2.975 x 10^9 CFU) were inoculated in 2.0 L of NB medium. After 48 h of culture growth, the bacterial cells (stationary-phase) were removed from the culture broth by centrifugation (1 000 × *g* for 10 min at 4ºC). A part (some) of the supernatant was stored at -80ºC for lyophilisation and further NMR spectroscopic analysis. The rest (residual) supernatant was maintained at -20ºC for accomplishing the remaining assays.

3.3. Antifungal activity of bioactive compounds

3.3.1. Antifungal paper disks diffusion assay

In order to evaluate the antifungal activity of the biological active compounds produced as described before in this chapter (Section 3.2), fungal spore suspensions of *Fusarium oxysporum, Aspergillus niger, Cladosporium* sp.*, Penicillium* sp.1*, Mucor* sp. *Rhodotorula* sp*, Penicillium* sp.2*, Penicillium* sp.3 *and Alternaria* sp. were prepared by adding a loopful of hyphae and/or spores from a MEA slant incubated at 25ºC for 7 days, to 5 mL of NaCl 0.85% solution. The suspension was filtered with sterilised cotton or triple gauze. For testing the antifungal activity, a 10^6 CFU/mL spore suspension was obtained through dilutions and fungal suspensions were incorporated in MEA at 45ºC 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 25ºC for 24–48 h. Commercial antifungal compounds (10 µL), Nystatin and Econazole (1 mg/mL) were used as positive controls, in order to compare the level of antifungal activity of these commercial antifungal drugs with those of the bioactive compounds. These compounds were also placed on the agar. The formation of inhibition halos around the discs indicates antifungal activity of the compounds tested and their diameter was considered as a measure of the sensitivity (Caldeira *et al.*, 2011a). The assays were performed in triplicate.

3.3.2. Interaction between novel biocides and heritage biodeteriogenic fungi

The interaction assays in liquid medium were also used for testing the antifungal activity of each biodeteriogenic fungi. The fungal suspensions previously used to carry out the antifungal paper disks diffusion assay (Chapter III, Section 3.3.1) were diluted and adjusted to a concentration of 10^5 CFU/mL. The interaction mixtures were prepared by mixing 5 mL of malt extract, 0.5 mL of 10^5 CFU/mL of spore suspension and 5 mL of culture broth and, then they were incubated for 24 h at 28ºC. Each interaction mixture (1 mL) was plated by incorporation in 20 mL of Cooke Rose Bengal agar (CRB agar - HIMEDIA), and the Petri dishes were incubated at 28ºC for 24–48 h. The relative inhibition was determined using the interaction test assays as well as a control test of the same fungus but in absence of the *Bacillus* sp. strains supernatants as reference. The relative inhibition was calculated as the quotient between the number of colonies counted in the test and in the control assays (Podile and Prakash, 1996; Caldeira *et al.*, 2008).

3.3.3. Statistical analyses

The results of the antifungal assays using the compounds produced by *Bacillus* strains, were evaluated statistically using the SPSS® 22.0 software for Windows

Copyright©, Microsoft Corporation (Annexe G). Descriptive parameters and One-way ANOVA were used in order to determine statistically significant differences at the 95% confidence level ($p<0.05$). The population variances homogeneity was confirmed by Levene test, being considered significant values whose probability of occurrence is greater than 95% ($p <0.05$).

3.2. Column chromatography

Antimicrobial compounds in lyophilised cell-free supernatant cultures were solubilised in $CHCl_3/CH_3OH/H_2O$ (65:35:5) and purified by flash column chromatography on silica gel (Merck, Kieselgel 60 with 0.040-0.063 mm) using the system $CHCl_3/CH_3OH/H_2O$ (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.

3.3. LC–ESI-MS analysis

The bioactive compounds solution obtained was filtered with a 0.45 µm nylon filter (VWR International, West Chester, PA, USA), and 10 µL of each fraction were analysed by liquid chromatography coupled to mass spectrometry (LC-MS).

LC-ESI-MS analyses were carried out in a LCQ Advantage ThermoFinnigan mass spectrometer equipped with an electrospray ionisation (ESI) source and using an ion trap mass analyser. The conditions of lipopeptides analysis, in positive mode were: capillary temperature 300ºC, source voltage 5.0 kV, source current 100.0 A, and capillary voltage 22 V. The mass spectrometer equipment was coupled to an HPLC system with auto sampler (Surveyor ThermoFinnigan). The analytical column was a reversed phase Zorbax Eclipse (C18, particle size 5.0 µm, 150 mm x 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 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 B (10–40 min).

3.4. Nuclear magnetic resonance (NMR)

^{1}H NMR spectra were performed on a Bruker Avance III HD 400 spectrometer at 400 MHz in DMSO-d6 (Euriso-top). ^{1}H shifts were reported relative to the ^{1}H signal of DMSO-d6 (δ=2.50 ppm) (Garbay-Jaureguiberry et al., 1978; Volpon et al., 2000).

3.5. Bioautographic detection

The lipopeptides produced were separated by thin layer reverse phase chromatography. The extracts in test (3 mg/mL) were applied (25 µL) on silica gel pre-coated TLC (Thin-Layer Chromatography) plates (0.2 mm, 20 x 20 cm, IF254, Merck, Darmstadt, Germany), eluted with $CHCl_3/CH_3OH/H_2O$ (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 by TLC, *Cladosporium* sp. cell suspension (10^6 cells/mL) was sprayed over the plates containing the detected compounds and was then incubated at 25ºC for 72 h, protected from light. The antifungal activity was indicated on the bioautograms by clear spots.

FTIR-ATR was used to monitor the chemical lipopeptidic nature and detect 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}.

4. Results and Discussion

Conservative interventions using direct and indirect methodologies have been applied in order to stop or slow-down the biodeterioration process. Biocide treatments are considered one of the practical approaches for bioremediation and conservation of artworks. The methodologies and products used must be chosen taking in mind the substrate conditions and the microbial population, looking for reducing the environmental impact and attending to avoid negative effects on/in the materials.

Many strains are known to suppress fungal growth due to the production of antifungal antibiotics (Leclère *et al.*, 2005; Mukherjee and Das, 2005; Hsieh *et al.*, 2008; Kim *et al.*, 2010a; Ben Slimene *et al.*, 2012; Mandal *et al.*, 2013a) especially cyclic lipopeptides such as surfactin, iturin and fengycin that can be used in versatile applications.

In this way, a methodological basis for the detection and characterisation of bioactive compounds produced by *Bacillus* strains have been proposed, for green biocide production directed to heritage biodeteriogenic fungi. The active compounds present in the bacterial culture broth have been characterised by a combined approach which include antifungal tests, NMR spectroscopy and MS spectrometry techniques.

Thereafter, in this chapter, is presented a simple, easy and fast combined methodology for carrying out a rapid evaluation of antifungal potential of the bacterial compounds and of their effectiveness for biocides production.

4.1. Antifungal activity of bioactive compounds

The antifungal activity of the supernatants from liquid cultures were tested against biodeteriogenic fungal strains previously isolated from historic wall paintings. The *in vitro* assays were carried out with high cells concentration of the following fungal strains: *Fusarium oxysporum, Aspergillus niger, Penicillium* sp.1*, Cladosporium* sp., *Mucor* sp.,

Rhodotorula sp*, Penicillium* sp. 2*, Penicillium* sp.3 and *Alternaria* sp.. The results obtained are summarised in Table III-1.

Table III-1. Antifungal activity of bacterial bioactive compounds and commercial antifungal drugs against the biodeteriogenic fungi, isolated from deteriorated mural paintings.

	Inhibition halos (mm)				
	***Bacillus* sp. CCLBH 1051**	***Bacillus* sp. CCLBH 1052**	***Bacillus sp.* CCLBH 1053**	**Nystatine***	**Econazole***
Aspergillus niger	21.2 ± 2.7	20.0 ± 0.3	21.4 ± 0.2	19.3 ± 0.6	18.1 ± 1.0
Fusarium oxysporium	12.8 ± 1.0	18.7 ± 0.7	12.7 ± 0.1	12.0 ± 0.1	14.0 ± 0.1
***Penicillium* sp.1**	21.4 ± 0.7	22.1 ± 0.5	20.0 ± 0.1	19.1 ± 0.3	21.1 ± 0.5
***Cladosporium* sp.**	22.1 ± 0.2	18.3 ± 0.1	37.4 ± 1.8	19.1 ± 0.2	21.0 ± 0.6
***Mucor* sp.**	25.7 ± 1.0	18.2 ± 1.0	27.3 ± 0.9	29.1 ± 1.1	31.8 ± 2.5
***Rhodotorula* sp.**	14.0 ± 0.1	14.5 ± 1.7	16.9 ± 0.1	20.1 ± 0.6	21.2 ± 0.7
***Penicillium* sp.2**	16.8 ± 0.7	13.0 ± 0.1	20.0 ± 1.0	16.8 ± 0.7	30.3 ± 8.3
***Penicillium* sp. 3**	23.7 ± 0.5	18.9 ± 1.0	26.7 ± 1.4	23.7 ± 0.5	59.7 ± 0.5
***Alternaria* sp.**	23.1 ± 0.9	17.9 ± 0.3	22.3 ± 0.7	23.1 ± 0.9	45.2 ± 0.4

***** Concentration of 1 mg/mL

The paper disk diffusion assay, using the supernatants of liquid culture corresponding to the stationary-phase of bacteria growth, showed higher inhibition level for the *Bacillus* sp. CCLBH 1053 cultures, independently of the fungal isolate tested.

The control assay do not show any inhibition halos around the discs. *Cladosporium* sp. was the most inhibited fungal strain by the compounds produced by *Bacillus* sp. CCLBH 1053. This was evidenced by the formation of an inhibition halo of 37.4 ± 1.8 mm (Figure III-1).

Figure III-1. Antifungal activity of biocides against *Cladosporium* sp. using the paper disk diffusion assay. A- Control. B- In the presence of 10 µL of *Bacillus* sp. CCLBH 1053 cell-free culture Broth.

Acceptable results were also obtained for *Bacillus* sp. CCLBH 1051 and CCLBH 1052, revealing high inhibitory capacity against the biodeteriogenic fungi tested, mainly for *Mucor* sp. and *Penicillium* sp.3, respectively. The only yeast tested, *Rhodotorula* sp., was the less inhibited fungal strain in this assay, showing the lowest halos diameter. This suggested a lower ability of the supernatants for inhibiting the proliferation of yeast. Whereas the majority of the fungal isolates were inhibited for the compounds produced by the three *Bacillus* strains, the level of inhibition depended strongly on the biodeteriogenic fungal strain tested. This indicates that for selecting the proper and most effective biocides to be applied for the conservation of artworks, it is crucial a previous identification and characterisation of the fungal communities responsible for the deterioration of the artwork to be treated.

For a better evaluation of the antifungal potential of the biocides produced, a comparative analysis with two commercial antifungal drugs Nystatine and Econazole (1 mg/mL) was carried out. These commercial compounds are known for their application in fungal infections treatment in mucous membranes in human beings.

When comparing the inhibition halos produced by the commercial compounds and the bacterial-synthesised compounds, significant differences were observed for some of the fungal strains tested.

Mixtures of compounds are frequently employed to allow an effective microbiological elimination, in order to avoid a quick recolonisation (Gaylarde *et al.*, 2011). Thus, for improving the biocide capacity of the compounds in analysis, a combination of the bacterial biocides could be considered in the future.

In order to confirm the antagonistic capacity of *Bacillus* bioactive compounds, an additional antifungal activity assay (liquid interaction assays) was performed. The solution resulting from the 24 h interaction of the antimicrobial agent and fungal spore suspension of preceding *Penicillium* sp.1, *Alternaria* sp., *Mucor* sp., *Fusarium oxysporium*, *Penicillium* sp.2, *Cladosporium* sp. and *Aspergillus niger*, were incorporated into agar medium.

The Figure III-2 exhibits the relative inhibition against a control test (%) where the fungi developed normally.

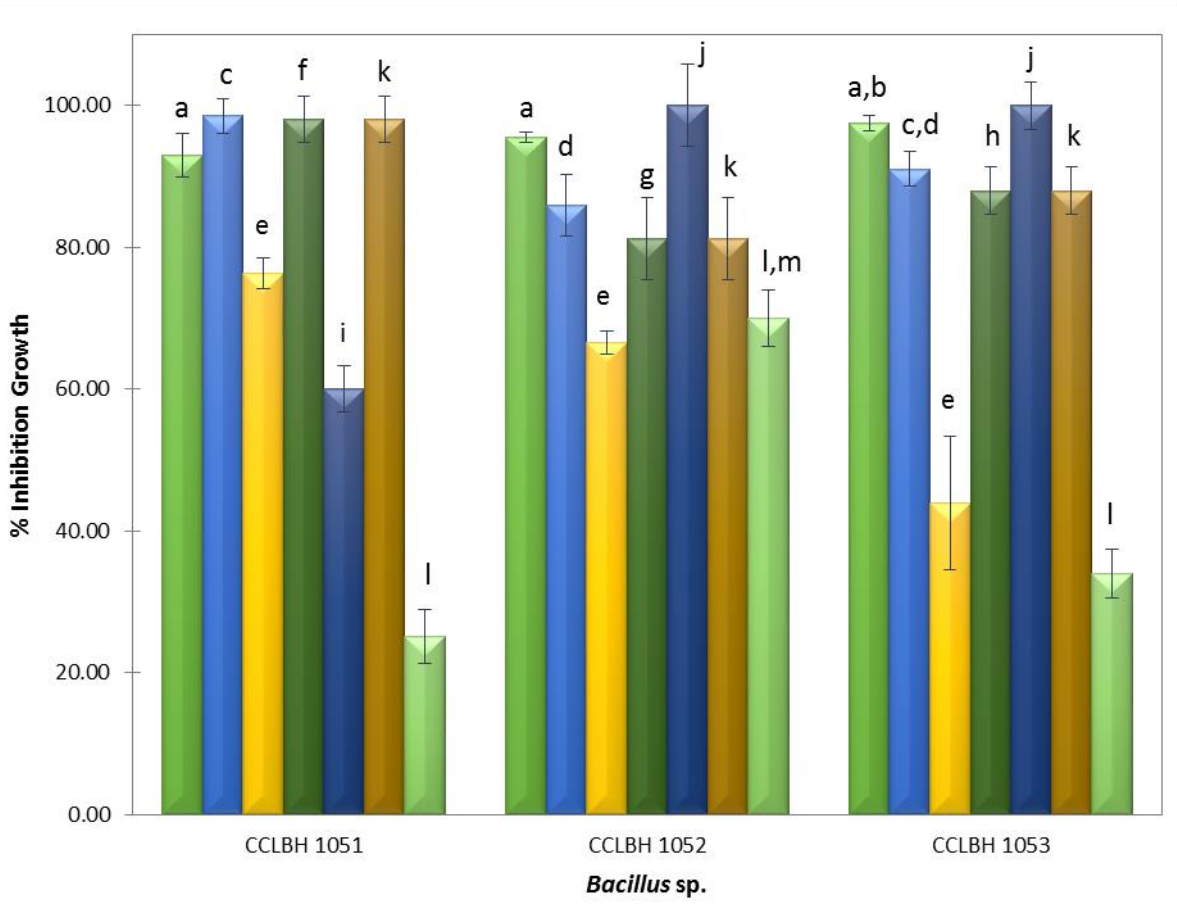


Figure III-2: Percentage of inhibition growth resulted from interaction assays between biodeteriogenic fungi and *Bacillus* sp. cell-free culture broth. (*Penicillium* sp.1, *Alternaria* sp., *Mucor* sp., *Fusarium oxysporum,* *Cladosporium* sp., *Penicillium* sp. 2, *Aspergillus niger).* Different letters (a-m) following the values indicate significant differences ($p<0.05$). Values of each determination represents means ± SD (n=3).

Once more, the results showed different levels of antifungal capacity depending on the biodeteriogenic target fungal strain tested.

The results of the interaction assay between novel compounds and heritage biodeteriogenic fungi, revealed that *Aspergillus niger* was the less inhibited fungus, nevertheless its inhibition release 70% when in interaction with supernatants of *Bacillus* sp. CCLBH 1052 cultures.

In contrast, *Penicillium* sp.1 was the most inhibited and consistent fungi tested, for all the compounds produced by the three *Bacillus* strains, with an average percentage of 95% inhibition. Also *Cladosporium* was highly inhibited by the compounds produced by *Bacillus* sp. CCLBH 1052 and CCLBH 1053 exhibiting a total inhibition (100%).

Particularly high antagonistic activity nearly to 100% was detected for all *Bacillus* cell-free supernatant of cultures, against *Penicillium* sp.1, *Alternaria* sp., *Mucor* sp., *Fusarium oxysporium*, *Penicillium* sp.2 and *Cladosporium* sp.. These outcomes confirmed the antifungal paper disk diffusion assay results previously obtained, which showed a high antifungal activity against the mentioned strains, but principally with *Penicillium* and *Cladosporium*. So, it has been proven that the new compounds produced by the bacterial strains *Bacillus* sp. CCLBH 1051, CCLBH 1052 and CCLBH 1053 inhibit strongly the heritage biodeteriogenic fungi tested.

Penicillium and *Cladosporium* were two of the most predominant fungi genera responsible for mural paintings and artworks colonisation (Sterflinger, 2010; Rosado *et al.*, 2014). Therefore, the confirmed inhibitory capacity against these specific fungal communities showed the high potential of these new natural compounds to prevent and suppress filamentous fungal proliferation in artworks. This fact, evidenced once more the great potential of this biotechnological approach applied to heritage context.

4.2. Characterisation of the antifungal compounds

To characterise the bioactive compounds produced by bacteria of the genera *Bacillus* several qualitative tests can be used (Das *et al.*, 2008; Caldeira *et al.*, 2011; Ben Slimene *et al.*, 2012; Silva *et al.*, 2015). A great number of peptides, antibiotics and proteins have been studied by NMR with the aim to elucidate the structure-biological activity relationships (Schneider *et al.*, 1999; Jiang *et al.*, 2000; Ruangwong *et al.*, 2012a; Pereira *et al.*, 2013). With the assistance of LC–ESI–MS spectral analysis it is possible

to infer about the presence of active lipopeptides in a culture broth mixture (Caldeira *et al.*, 2011b).

Results obtained accomplish a ground-breaking research for the potential of these compounds on heritage materials contaminated by fungal communities, opening the door for a novel green alternative for chemical biocides commonly used.

A new methodological scheme using analytical approaches was established in order to access and characterised bioactive metabolites produced by *Bacillus* strains, without previous isolation.

The LPP produced by *Bacillus* sp. CCLBH 1051, CCLBH 1052 and CCLBH 1053, previously separated by a flash silica gel column were analysed by LC-ESI mass spectrometry (Figure III-3 to Figure III-5).

Figure III-3 shows the chromatogram of the supernatant liquid culture of *Bacillus* sp. CCLBH 1051 and its mass spectra.

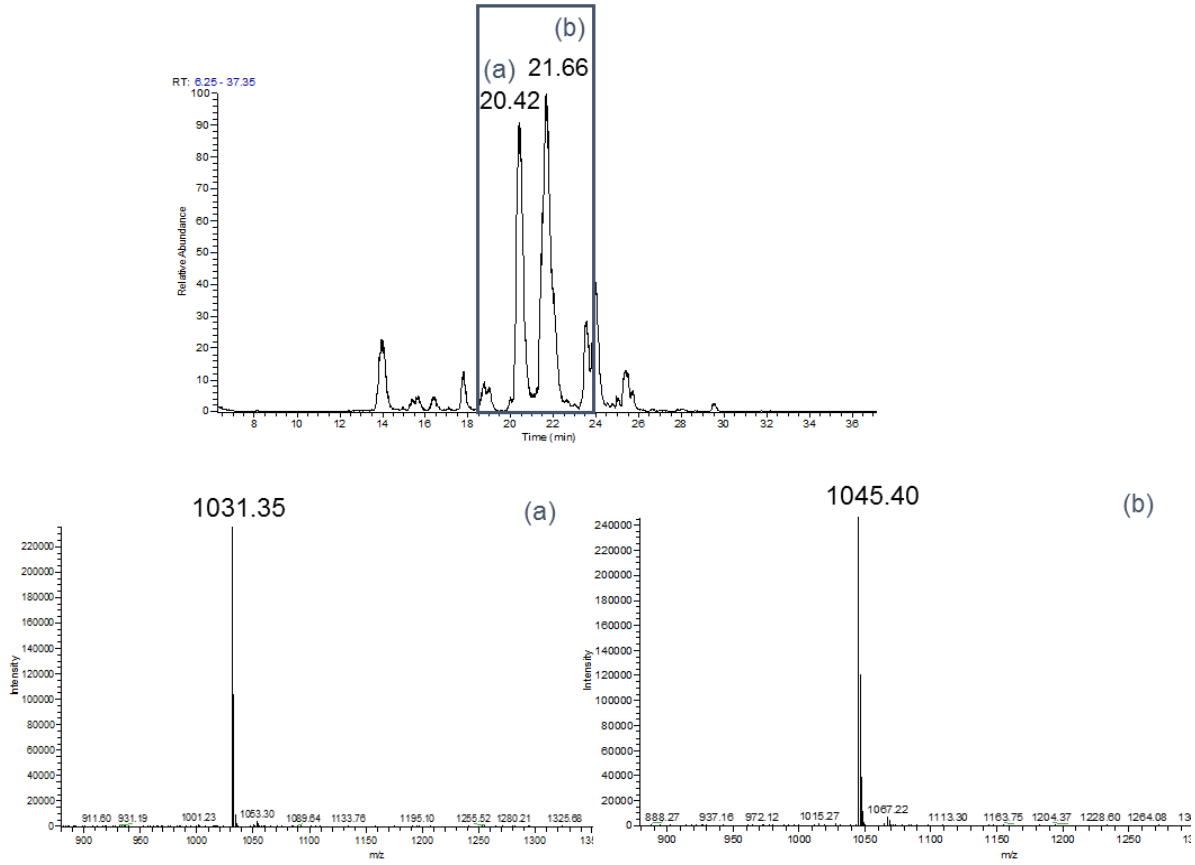


Figure III-3: Total ion chromatogram of the supernatant liquid culture of *Bacillus* sp. CCLBH 1051 and mass spectra corresponding to the peaks (a) m/z 1031.35 and (b) m/z 1045.40.

The chromatogram and mass spectra corresponding to the supernatant liquid culture of *Bacillus* sp. CCLBH 1052 are shown in Figure III-4.

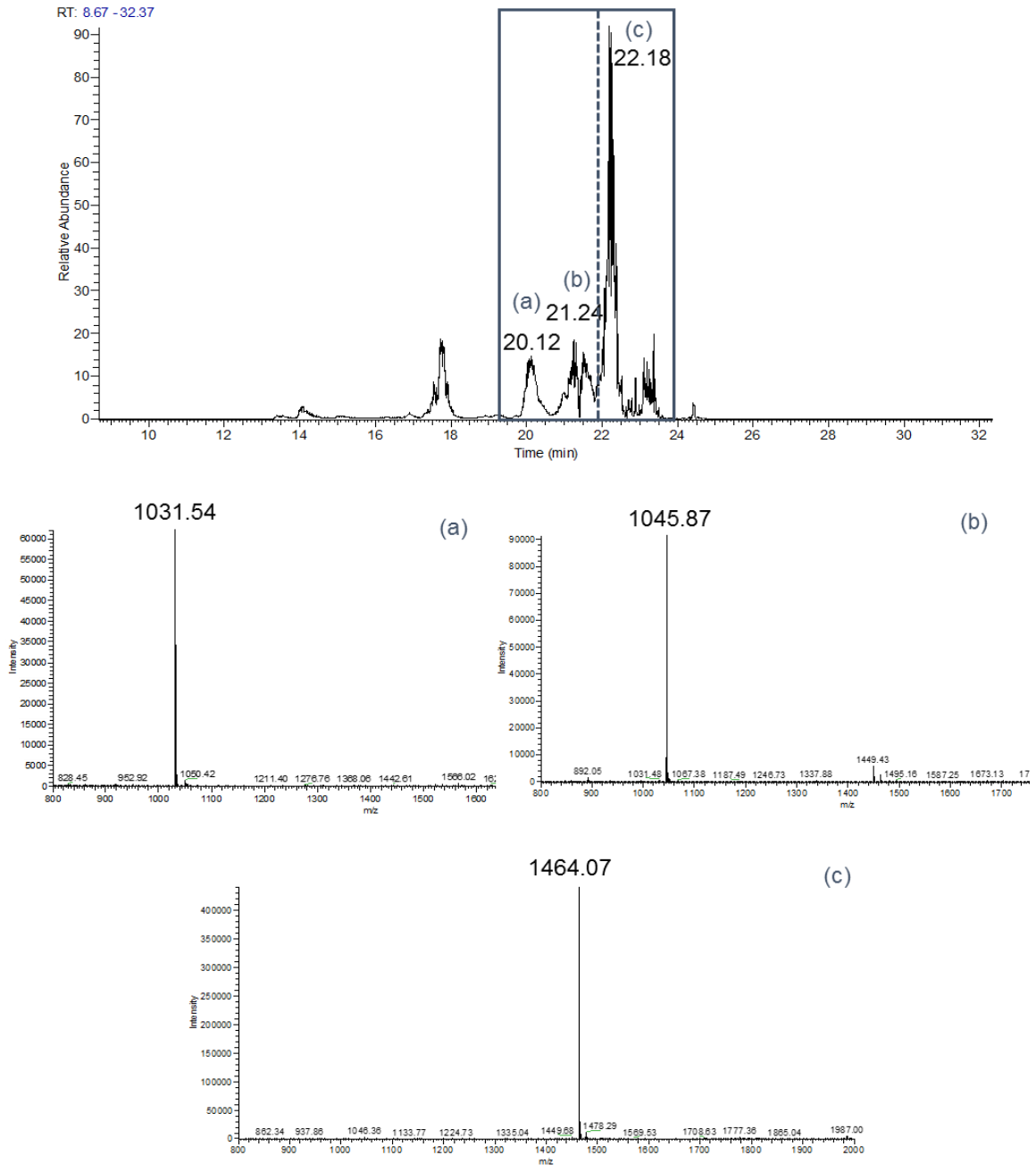


Figure III-4: Total ion chromatogram of the supernatant liquid culture of *Bacillus* sp. CCLBH 1052 and mass spectra corresponding to the peaks (a) m/z 1031.27, (b) m/z 1045.31 and (c) m/z 1464.07.

In the same way, Figure III-5 show the chromatogram of the supernatant liquid culture of *Bacillus* sp. CCLBH 1053 and the mass spectra obtained for some of the detected peaks.

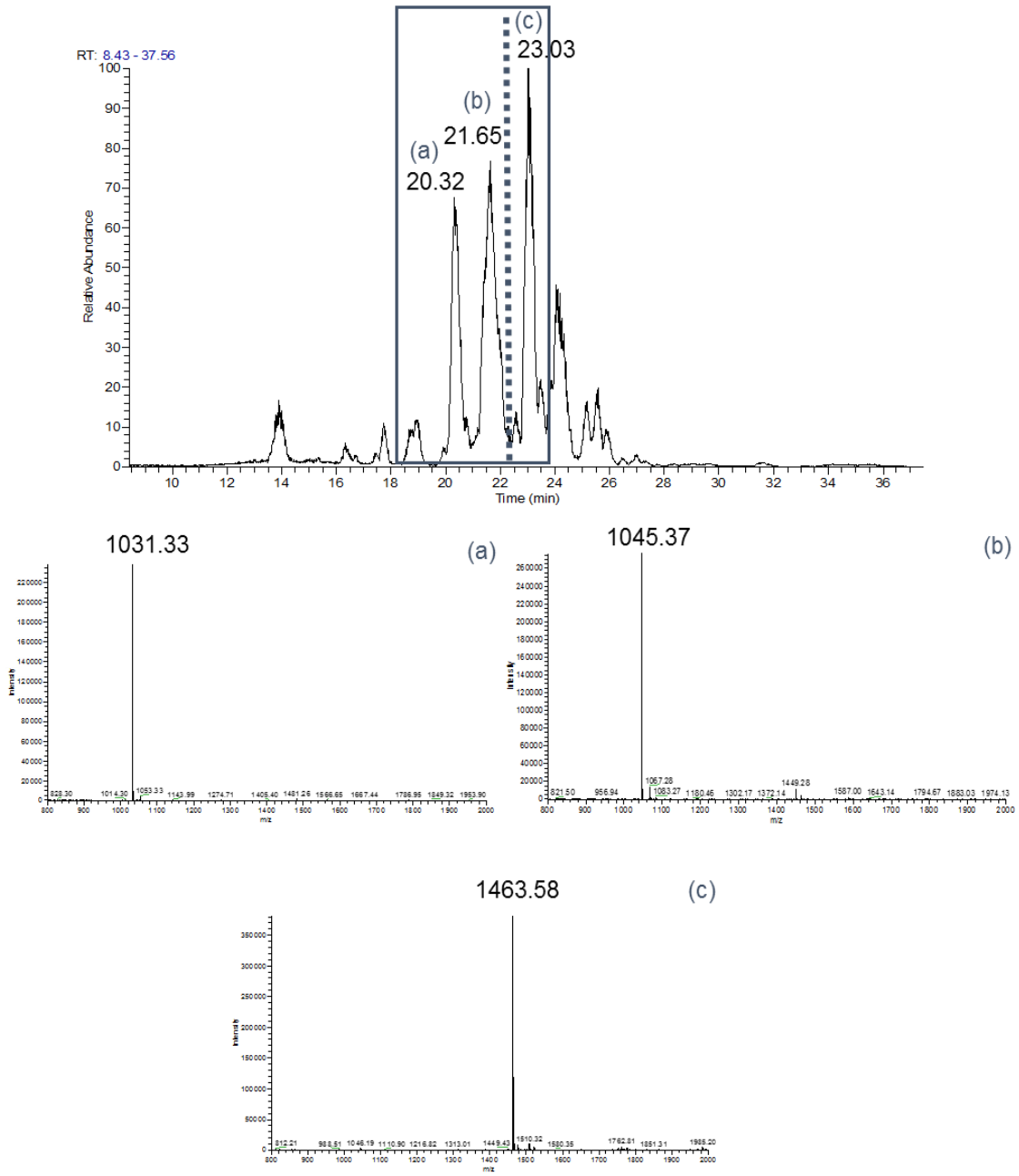


Figure III-5: Total ion chromatogram of the supernatant liquid culture of *Bacillus* sp. CCLBH 1053 and mass spectra corresponding to the peaks (a) m/z 1031.37, (b) m/z 1045.37 and (c) m/z 1463.53.

The LC–ESI–MS spectral analysis of all the *Bacillus* sp. cell-free supernatant showed two main clusters containing molecules that were observed at m/z 1031 and 1045. These clusters, according with previous studies may belong to putative iturin class (Kim *et al.*, 2010a; Caldeira *et al.*, 2011b; Mandal *et al.*, 2013a).

Bacillus sp. CCLBH 1053 exhibits also peak at m/z 1463 that may correspond to putative fengycin class, according with the same authors. This peaks differ by 14 Da suggesting a series of homologous molecules with different lengths of fatty acid chain (i.e., CH_2=14 Da).

This antifungal compound belongs to a family of lipopeptides which can be extracted from the culture media of various strains of *B. subtilis* (Maget-Dana and Peypoux, 1994; Ruangwong *et al.*, 2012b).

In the literature, bacterial lipopeptides such as iturin A, surfactin and fengycin exhibited the same peak profile that the obtained with this strains. 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 (Kim *et al.*, 2010a). Caldeira *et al*. (2011) reported that *Bacillus amyloliquefaciens* CCMI 1051 exhibit high levels of antagonistic properties against filamentous fungi due to the production of compounds with masses between 1000 and 1100 Da, comparable to iturin and surfactin compounds, and between 1436 and 1478 Da, compatibles to fengycin (Caldeira *et al.*, 2011b).

Thus, the peaks with m/z 1031, 1045 and 1463 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. (Caldeira *et al*., 2008). In fact, several isoforms exist for each lipopeptide due to diverse producer strains and different nutritional conditions. These parameters affect the substitution of amino acids in the peptidic ring and the length of lipidic chain (Leenders *et al.*, 1999), that were possible to observed in the LC-MS results obtained.

Additionally, their chemical analysis by ^{1}H-NMR was performed in order to access a rapid detection methodology for the antifungal compound in cell-free supernatant of *Bacillus* strain. The results are shown in Figure III-6.

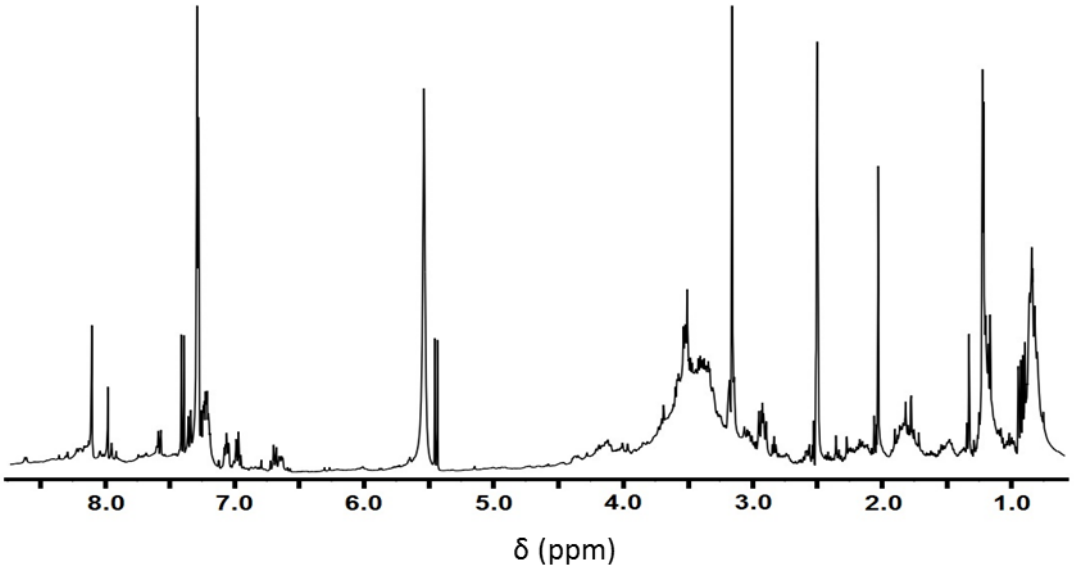


Figure III-6: ^{1}H-NMR spectra of the bioactive compounds produced by *Bacillus* sp. CCLBH 1053.

The ^{1}H-NMR spectra of the bioactive compounds produced by the three strains of *Bacillus* show the same characteristics and profile. Only the correspondent to the analysis of the bioactive compounds produced by *Bacillus* sp. CCLBH 1053 are shown, as example, as they are the most representative.

Figure III-6 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. 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 ~ 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 (constituted by amino acids and a fatty acid chain) (Volpon *et al.*, 2000; Tang *et al.*, 2007; Ma *et al.*, 2012;

Ruangwong *et al.*, 2012a), suggesting the presence of bacterial lipopeptides in the cell-free culture broth supernatant.

4.2. Bioautographic methodology for screening the bioactive compounds

In order to develop a rapid and simple methodology for detection of bioactive compounds produced by *Bacillus* strains, the compounds from *Bacillus* sp. CCLBH 1053 cultures (the most efficient produced strain), were separated by thin layer reverse phase chromatography and used in a bioautographic detection. FTIR-ATR analysis were used to monitor the chemical lipopeptidic nature of the biologically active compounds on the bioautographic assays against *Cladosporium* sp., the fungal strain inhibited with more efficiency in the previous antifungal assays.

Figure III-7 shows the chromatographic scheme profile (Figure III-7A) and the bioautogram correspondent, obtained from the chromatographic plate (Figure III-7B) and from the cropped spots (Figure III-7C).

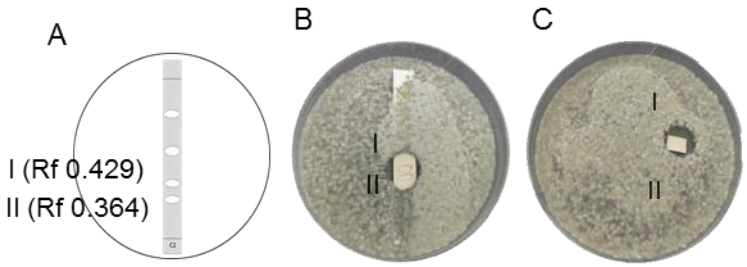


Figure III-7: Autobiographical results. Chromatographic scheme profile. A- The *Bacillus* sp. CCLBH 1053 extracted cell-free supernatant shows the presence of two antifungal compounds against biodeteriogenic fungi *Cladosporium* sp. B –Scheme of TLC plate in Petri dish with the two layers (I- Rf = 0.429 and II- Rf = 0.364 imagem); C- Bioautograms with TLC layers applied separately from *Bacillus* CCLBH 1053.

TLC shows layers with different Retention Factor (RF) detected on a UV chamber at 254 nm and 365 nm, however just two spots show antifungal activity in autobiographical assay (I- Rf = 0.429 and II- Rf = 0.364). The clear spots with Rf

values of 0.429 (I) and 0.364 (II) corresponds to the antifungal compounds produced by CCLBH 1053 strain. In fact, in Figure III-7 it is notorious the capacity of the chromatographic separated compounds to inhibit the development of *Cladosporium* sp. fungal strain, showing once more the ability of these strains to produced biological active compounds with antifungal potential.

FTIR-ATR analysis, of the two spots separated by TLC that showed antifungal capacity, were immediately performed, in order to clarify and confirmed the chemical nature of the compounds (Figure III-8).

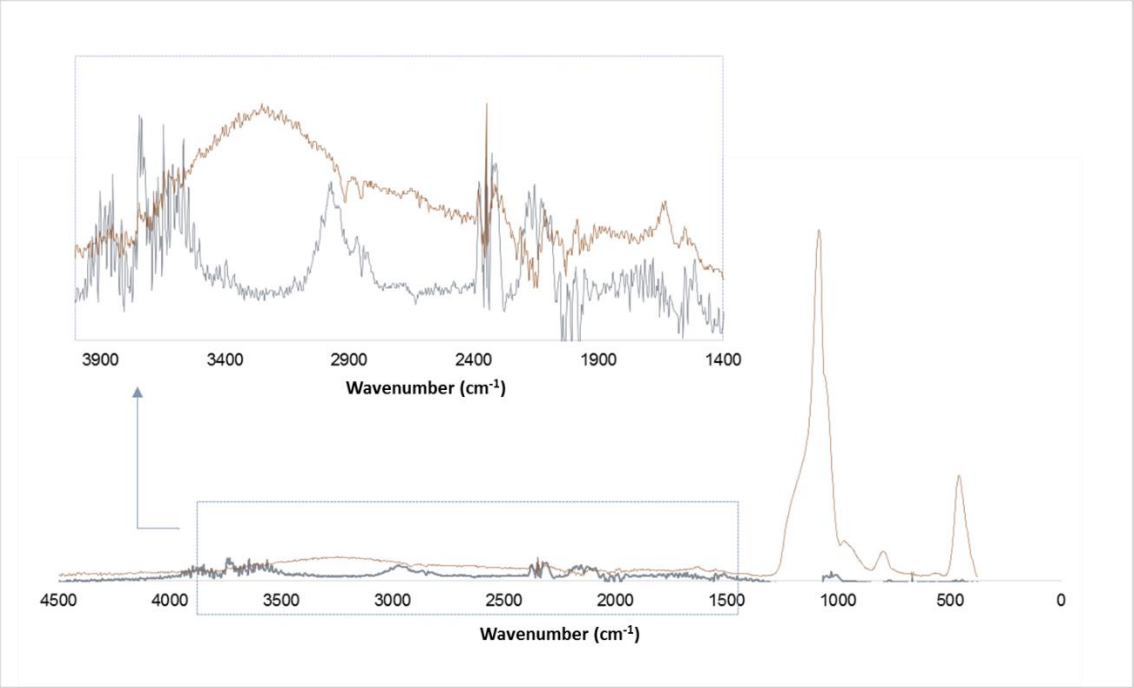


Figure III-8: FTIR-ATR analyses of the biologically active compounds produced by *Bacillus* CCLBH 1053 (I- Rf = 0.429) on the bioautographic plate. (—— *Bacillus* sp. CCLBH 1053; —— Silica)

The FTIR-ATR spectra, corresponding to clear spot that proved to have antifungal capacity (I-Rf = 0.429), showed a broad absorbance peak around 3645 cm^{-1}, ranging from 3500 to 3740 cm^{-1}.

These peak constitute a typical feature of compounds containing carbon and amino groups and 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 and the peak at 2980 cm^{-1}, indicates the presence of C–CH_3 bonding or long alkyl chains typical of lipopeptide compounds (Das *et al.*, 2008a; Varadavenkatesan and Murty, 2013). The peak in the region from 1520 to 1555 cm^{-1}, corresponds to C=O bonds and its stretching vibrations. FTIR-ATR spectra also revealed a more evidenced peak at 1090 cm^{-1} which corresponds to the silica background, present in the matrix of TLC plate.

These FTIR-ATR findings allow to establish that the antifungal compounds produced possess a lipopeptidic nature, being consistent with the results obtained by LC-ESI-MS and NMR analysis, revealing yet to be useful for the characterisation of this kind of compounds. In fact, FTIR-ATR analysis constituted an added value for the development of a combined methodology for the rapid lipopeptide detection and chemical nature identification.

5. Conclusions

The *Bacillus* sp. CCLBH 1051, CCLBH 1052 and CCLBH 1053 strains produce compounds that have the ability to suppress growth of heritage biodeteriogenic fungal isolates. A combined methodology, using antifungal tests, spectroscopic (NMR and FTIR-ATR) and chromatographic techniques (LC–ESI-MS) complemented with bioautographic methodology, could detect and evaluate their antifungal potential. These results allowed to achieve a rapid detection of the bioactive compounds as well as of their antifungal potential, without the need of previous total isolation.

The analysis of the supernatant from the *Bacillus* sp. CCLBH 1051, CCLBH 1052 and CCLBH 1053 cultures showed the real potential of these compounds to reduce and control the growth of fungi involved in artworks biodeterioration process.

LC-MS-ESI revealed the presence of compounds with masses of 1031 and 1045 Da, compatible to iturin family which are produced by *Bacillus* sp. CCLBH 1051, CCLBH

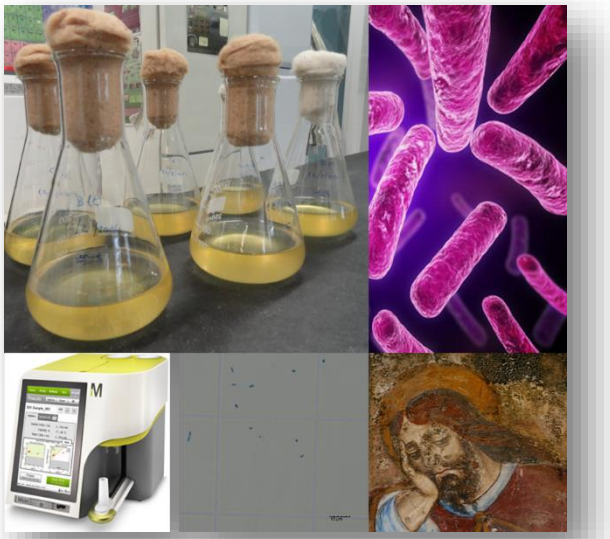
1052 and CCLBH 1053. Additionally *Bacillus* sp. CCLBH 1053 also produces compounds between 1463 and 1477 Da, suggesting the formation of fengycin lipopeptides family as product of its secondary metabolism. Also, the lipopeptidic nature of the compounds was confirmed by ^{1}H-NMR and FTIR-ATR analyses, showing the peptide bonds and methylene protons of long aliphatic chains in the lipopeptide molecule.

The combined methodology developed and described here allowed to access a fast screening of the bioactive metabolites produced without need of the laborious total previous isolation. Therefore, this approach can be employed for monitoring the antifungal compounds production for yield optimisation.

These bioactive compounds represent a powerful alternative that could be used as additives in new formulations of novel biocides production, constituting an efficient and environmental safe solution to face the problem of biodegradation/biodeterioration of cultural heritage.

CHAPTER IV

Physiology of *Bacillus* with antifungal properties: Lipopeptides production



1. Overview

The production of lipopeptides (LPP) after a starvation period is a natural phenomenon that occurs in some *Bacillus* species. The knowledge about the factors that trigger the production of antifungal LPP compounds by *Bacillus* strains and the factors underneath their production are vital in the search of alternative biological means to act against biodeteriogenic fungi that promote biodeterioration of built culture heritage.

Therefore, this study constitutes a breakthrough research which can lead to be considered on scale-up of the production process for future applications as a new natural biocide. This chapter intends to clarify the physiological phenomena underneath the bioactive compounds production, using a ground-breaking methodology for the physiological response of *Bacillus* strain to nutrient starvation and biological active compounds production monitorisation study.

2. Introduction

In recent years, the interest in using *Bacillus* sp. strains as biocontrol agents has considerably increased, as they produce a wide variety of antimicrobial substances. Sporulation of some *Bacillus* species, a natural phenomenon that occurs after a starvation period, has been previously associated with the production of LPP in liquid cultures (Rahman *et al.*, 2006). Whereas the mechanisms involved are not yet well established, it has been pointed out that it can be associated to the fact that, some genes of the sporulating killing factor operon seem to present great similarity to genes involved in the regulation of peptide antibiotics biosynthesis and the signalling of the sporulation gene product Spo0A (González-Pastor *et al.*, 2003; Piggot and Hilbert, 2004). And, in some way, sporulation and biosynthesis of peptide antibiotics seems to be activated in response of the same stimuli.

As other microorganisms, *Bacillus* species respond to nutritional limitation by entering a resting state which can remain dormant for many years. The formation of this robust resting cell, the endospore, is the result of a series of changes in the transcription pattern. As endospore formation is an elaborate and energy intensive process that requires several hours to complete (Piggot and Hilbert, 2004; Rahman *et al.*, 2006), bacteria could be expected to delay it until they are forced to do so by prolonged depletion of nutrients (González-Pastor *et al.*, 2003).

Whereas the link between antibiotic production and *Bacillus* sporulation is not fully understood, it is attributed, among other factors to the stage of the culture. Though their productions are carried out at different growth phases during the culture incubation, the resting stage-sporulation finally ends in these lipopeptides production.

Thus, the parallel investigation of the cells physiology (evolution along time of cell viability and concentration, both of spores and cells), spores concentration evolution and lipopeptides production can help to elucidate and to deep the knowledge of the association between sporulation and lipopeptides production.

The assessment of cell viability (i.e. the number of healthy cells in a sample) may be important in assessing the progress of a cell culture and the drug- or cell-mediated cytotoxicity (Davey, 2011). A bacterial cell is generally considered “viable” if it possesses all the components and mechanisms necessary for sustained proliferation. For the cell viability assessment, in spite of its restrictions, the direct counting of stained cells under microscopic observation is a simple method that can be adopt. However, other methods that allows to overcome these limitations can be used, including image analysis and flow cytometry (Davey, 2011; Kim *et al.*, 2016).

A flow cytometer allows to detect and quantify physical parameters, such as electrical impedance, light scattering and fluorescence intensity (Sutherland *et al.*, 2012). These parameters are related or can be correlated to cellular parameters. In this way, flow cytometry allow to collect several parameters, such as cell size, cell membrane properties, cytoplasmic constituents, cell organelles, DNA content and nuclear chromatin, among others (Müller *et al.*, 2010; Rieger *et al.*, 2011). By the judicious selection of proper and compatible cocktails of fluorescent dyes and probes, multiparametric measurements can be used to quantify the uptake of fluorescent dyes and, in this way, to discriminate subpopulations of cells according to characteristics of interest (Marahier *et al.*, 1993; Comas-Riu and Vives-Rego, 2002; Müller *et al.*, 2010).

Thus, flow cytometry has been used, among others, for analysing bacteria sporogenesis and is one of the most common and straightforward ways to measure cell viability and assess cytotoxic consequences, by assessing cell membrane integrity. These viability stain-based methods are based on the exclusion and uptake of fluorescent or fluorogenic stains (Davey, 2011; Kwolek-Mirek and Zadrag-Tecza, 2014). The mechanism of action of the dyes used for assessing the viability depends on the properties of the cell membrane and is based on the supposition that cell membrane damage leads to cell death.

The stains used may be divided into two subcategories: i) Dyes that are excluded by the intact membranes of viable cells (Christiansen *et al.*, 2003); ii) Dyes that penetrate both into alive and dead cells. In combination with these dyes it is usual to use nucleic-acid specific dyes that allow to detect and distinguish cell from particles (Davey, 2011). Viability-stain methods provide information only about death or life cells.

In this chapter, for investigating the evolution of cells viability along time, a combination of two dyes was used: Annexin V and 7-aminoactinomycin D (7-AAD). Annexins are a family of calcium-dependent phospholipid-binding proteins, which bind to phosphatidylserine (PS) to identify apoptotic cells. This staining method, paired with 7-AAD or propidium iodide (PI) is widely used to identify apoptotic stages or necrotic through differences in plasma membrane integrity and permeability by flow cytometry (Kaprelyants and Kell, 1992; Davey, 2011).

Due to the multiple advantages that flow cytometry provides, this technique in combination with biochemical methods has been used to reveal the heterogeneity of *Bacillus* cultures (Chung *et al.*, 1995; Comas-Riu and Vives-Rego, 2002) and, more recently, to distinguish between live and dead endospores on the basis of their cytometric scatter (Stopa, 2000). However, flow cytometry studies on sporogenesis and spore sorting in *Bacillus* cells remain rare.

The development of an efficient bioprocess with low production costs is extremely important for commercial production of lipopeptide antibiotics (Jin *et al.*, 2015). With the intention of improving product yields/concentrations, several authors have described different culture conditions and procedures for optimising cultivation conditions (De Bruijn and Raaijmakers, 2009; Leães *et al.*, 2013).

Secondary metabolites including lipopeptides of the iturin group are produced after the logarithmic cell growth phase when one or more essential nutrients become undersupplied, as described in the previous chapter. Lipopeptide synthesis seems to be regulated by mechanisms associated with starvation processes and closely linked to cells sporulation. Some authors already report that *Bacillus* sp. sporulation phenomenon

might occur due to a response to starvation that will eventually end with the production of antibiotic compounds such as lipopeptides (Marahier *et al.*, 1993; Piggot and Hilbert, 2004; Rahman *et al.*, 2006; Caldeira *et al.*, 2008; Jin *et al.*, 2015).

The purpose of this chapter was to monitor the physiology of the *Bacillus* sp. CCLBH 1053 cells by multi-parameter flow cytometry and the physiological response of this microorganism to nutrient starvation and new supplementation, evaluating at the same time the bioactive compounds production.

3. Materials and Methods

3.1. Microorganism and culture media

The *Bacillus* sp. CCLBH 1053 with antifungal potential (results shown in previous chapter) were maintained on NA (Nutrient Agar) slants and stored at 4ºC. Cells were suspended in physiological serum and 2.975 x 10^9 cells were used to inoculate 100 mL of NB and LAPM (Lipopeptide Antibiotic Production medium, composition in Annexe A) media.

3.2. *Bacillus* growth monitoring

To monitor the *Bacillus* sp. CCLBH 1053 cells growth, liquid cultures of NB and LAPM media were incubated for 720 h at 30ºC in an orbital shaker at 120 rpm (IKA KS 4000 I control) and the absorbance was periodically monitored at 600 nm.

3.3. Spores formation assessment

The *Bacillus* sporulation were analysed by two different techniques: i) by platting the culture on NA plates and counting the number of CFU (Colony-Forming Unit); and ii) by flow cytometry, analysing the forward scatter (related to the cell size).

For CFU counting, aliquots (2.0 mL) of NB and LAPM culture medium were removed from the culture under aseptic conditions at 0, 5, 24, 48, 72, 96, 120, 144, 168, 192, 220, 264, 360, 432, 528, 720 h of culture growth. The samples were prepared in triplicate and heated at 77ºC for 12 min in a water bath. The concentration of *Bacillus* spores (CFU/mL) was determined after plating the medium on nutrient agar in a serial dilution procedure and incubation for 24 h at 30°C. Counted plates were only considered when the number of CFU in a plate were between 30 to 300 CFU.

For the flow cytometry analysis, a Muse™ Cell analyser (Millipore, Darmstadt, Germany) was used. It is a flow cytometer equipped with a 532-nm green laser, a photodiode detector for forward scatter detection and two different photodiode detectors for yellow and red fluorescence detection (576/28 and 680/30, respectively). Each sample was analysed in triplicate and for each replicate 2000 events were acquired. The settings of the equipment were adjusted by analysing the blanks and the corresponding samples. Flow cytometry acquisitions were performed using PBS/BSA 1% (w/v) as sheath fluid.

The results obtained were analysed using the MuseSoft 1.4.0.0 and expressed as cells concentration (cell/mL). Data were additionally analysed with Flowing software version 2.5.1 Turku Centre for Biotechnology, University of Turku, Finland.

3.4. Cell monitoring physiology

Bacillus sp. CCLBH 1053 cell physiology monitorisation was carry out by flow cytometry using the Muse™ Annexin V & Dead Cell kit (Millipore, Darmstadt Germany). The evolution along time of the live, early and late apoptotic cell concentrations, as well as the concentration of cells that have died through non-apoptotic pathway in the culture was followed.

For the staining procedure aliquots (2.0 mL) of the culture were collected in 2.0 mL centrifuge microtubes at 0, 5, 24, 48, 72, 96, 120, 144, 168, 192, 220, 264, 360, 432,

528, 720 h of culture growth. After centrifugation, the supernatant was transferred to another 2.0 mL microtube and maintained at -20ºC for further LC-ESI-MS analysis and the pellet were resuspended in BSA/PBS 1% (w/v). Cell concentration was determined by cell counting with Neubauer chamber.

For each staining assay, the volume of cellular suspension containing 8 x 10^4 cells were added and made up to 100.0 µL with BSA/PBS 1% (w/v). After that, 100.0 µL of the Muse™ Annexin V & Dead Cell Reagent was added and the samples incubated at 25ºC for 20 min in the dark. For each assay, duplicates of the samples were prepared and the correspondent blank was also analysed (it consists on avoiding the use of Muse™ Annexin V & Dead Cell Reagent. Thus, instead of this reagent 100.0 µL BSA/PBS 1% (w/v) were added to each blank).

The flow cytometry analysis was performed using a Muse™ Cell Analyser as described in Section 3.3.

The results obtained were analysed using the MuseSoft 1.4.0.0 and expressed as cells concentration (cell/mL) in each quadrant; viable cells, [Annexin V-PE (–) and dead cell marker (–)]; cells in the early stages of apoptosis [Annexin V-PE (+) and dead cell marker (–)]; cells in the late stages of apoptosis or dead by apoptotic mechanisms [Annexin V-PE (+) and dead cell marker (+)]; and cells that have died via necrosis but not through the apoptotic pathway [Annexin V-PE (–) and dead cell marker (+)]. Data were additionally analysed with Flowing software version 2.5.1 Turku Centre for Biotechnology, University of Turku, Finland.

3.5. Monitorisation of LPP production

For monitoring the production of lipopeptides along time in the liquid cultures of NB and LAPM media, LC-ESI-MS analysis of the supernatants, obtained at different times after centrifugation of aliquots (2.0 mL) of the culture were performed. They were collected and frozen as described in Section 3.4. Once all were collected, they were

defrosted and filtered with a 0.45 µm nylon filter (VWR International, West Chester, PA, USA). Aliquots (10 µL) of the filtered sample were analysed by liquid chromatography coupled to mass spectrometry (LC-MS).

LC-ESI-MS analysis were carried out in a LCQ Advantage ThermoFinnigan mass spectrometer equipped with an electrospray ionization (ESI) source and using an ion trap mass analyser. The conditions of lipopeptides analysis were: capillary temperature 300ºC, source voltage 5 kV, source current 100 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 x 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 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 B (10–40 min).

3.6. Spores germination of *Bacillus* sp. CCLBH 1053

Figure IV-1 shows the methodological scheme used for inducing spores germination. Three set of assays were conducted in parallel:

- The NB assays operated as a control, using NB medium for culture growth (100 mL of NB medium inoculated with *Bacillus* sp. CCLBH 1053 cells, incubated at 30ºC and under continuous shaking, 120 rpm).

- Two assays inducing germination of spores that were cultures prepared as the control but that at 72 h were: i) supplemented with fresh Peptone previously sterilized (8% of culture volume, w/v) and incubated again under the same conditions (Nutrient Broth Peptone Supplementation assay or NBPS); or ii) supplemented with Peptone and followed by a sub-lethal heat shock of 77ºC for

12 min to activate the spores (Nutrient Broth Peptone Supplementation and Heat-Activation assay, NBPSHA).

Powdered Peptone (HIMEDIA) was sterilized at 120ºC for 20 min. The sterilization efficiency was confirmed by spreading an aliquot of the medium on the NA plate. None colony appeared after 24 h.

The spore concentration in initial samples for the different times of culture before and after induction of spore germination were obtained by counting the number of CFU incorporated on NA plates. Additionally, the bacteria cells distribution was analysed by flow cytometry on the basis of their forward scatter (related to the cell size) as described in Section 3.3. For additional information total cell concentration in both assays was determined by cell counting with Neubauer chamber and the quantity were expressed as [cell]/[spore] ratio.

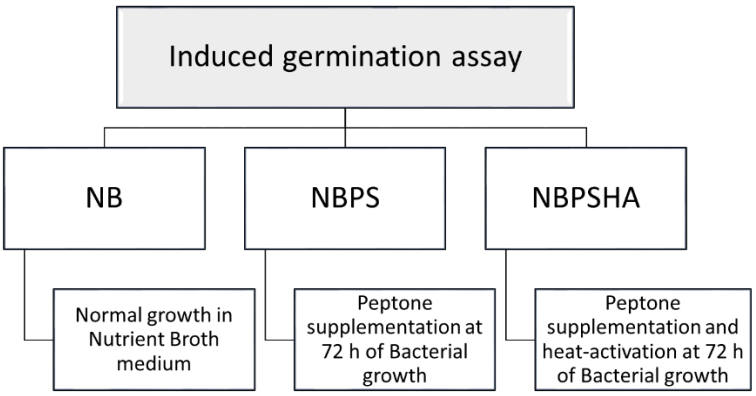


Figure IV-1: Methodological scheme of inducing germination assay.

LC-ESI-MS analysis were performed in order to obtain the relative quantification of the bioactive compounds in the different assays, as described in Section 3.5.

4. Results and Discussion

The association between the antibiotic production and *Bacillus* sporulation is not fully understood. Some authors have referred antifungal activity and sporulation as linked phenomena (Dieckmann *et al.*, 2001; Yazgan *et al.*, 2001). However, some studies with mutant strains described sporulation without antibiotic formation (Leifert *et al.*, 1995) and others an sporulation increase when antibiotic production decrease (Chevanet *et al.*, 1986).

It is important to understand how differently *Bacillus* sp. strains respond to the environmental alterations, how such responses may be accurately monitored and measured and essentially how could affect the production of metabolites of interest such as antimicrobial compounds.

In this chapter, the correlation between the growth profile, the sporulation phenomena and the production of bioactive compounds were study in order to characterise, interpret and understand the LPP production and the relation with cell viability and sporulation. From all the bacteria investigated in this work, *Bacillus* sp. CCLBH 1053 was selected for this study due to its remarkable potential to produce antifungal compounds, as compared with the rest of the bacteria strains used in the previous chapters.

4.1. Cell growth dynamics of *Bacillus* sp. CCLBH 1053

Absorbance measurements at 600 nm is indicative of microbial growth and provide an initial information of cell proliferation state.

In order to compare the time course profile of the *Bacillus* sp. CCLBH 1053 in two different liquid culture media NB and LAPM (Rahman et al., 2006), absorbance measurements at 600 nm (A600) were monitored along time. Figure IV-II shows the results obtained.

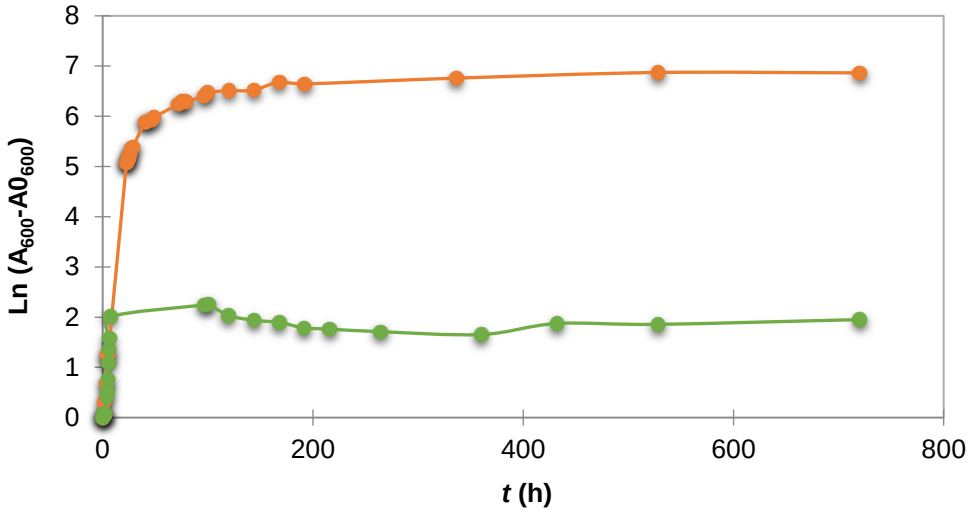


Figure IV-2: Time course profiles of *Bacillus* sp. CCLBH 1053. NB medium (●); LAPM medium (●). All data were determined in triplicate.

The time course profile of *Bacillus* sp. CCLBH 1053 in NB medium obtained show a lag phase of about 2 hours, a period of exponential growth of approximately 8-10 hours and a stationary phase that is reached after 11 hours of culture (Figure IV-2). However, with the LAPM medium the time course profile shows a further delay where the lag phase extends to 20 hours and the stationary phase begin after 51 hours of culture (Figure IV-2).

Due to its higher peptone content, the LAPM medium produces higher cell concentrations at each time, as well as shows a longer stationary phase than the NB medium.

4.2. *Bacillus* sporulation assessment

In this work a comparative study of the evolution sporulation profiles and cell distribution of *Bacillus* sp. CCLBH 1053 strain along time, in two distinct culture media, NB and LAPM, was performed along 720 h (Figure IV-3).

Figure IV-3 shows a slight increase in spore concentration during the first 96 h independently of the media used for growth. In a second phase, a strong increase of the spore concentration was observed from 96 to 168 h for NB, and from 120 to 408 h for

LAPM medium, when they reached their maximum value. For the last medium a third phase was observed that is characterised by a decline of spore concentration after 408 h of culture growth. Nevertheless, for the NB medium after a strong decline of the spore concentration, from 168 to 264 h, a slight increase is observed, from 264 to 432 h of incubation, followed by a new decline.

A higher concentration of spores is produced in the NB medium (Figure IV-3B), but their formation is notably slower in LAPM media. This can be related to the fact observed when comparing the growth curves, with the LAPM medium the culture stays in a stationary stage for a longer time than with the NB medium (Figure IV-2).

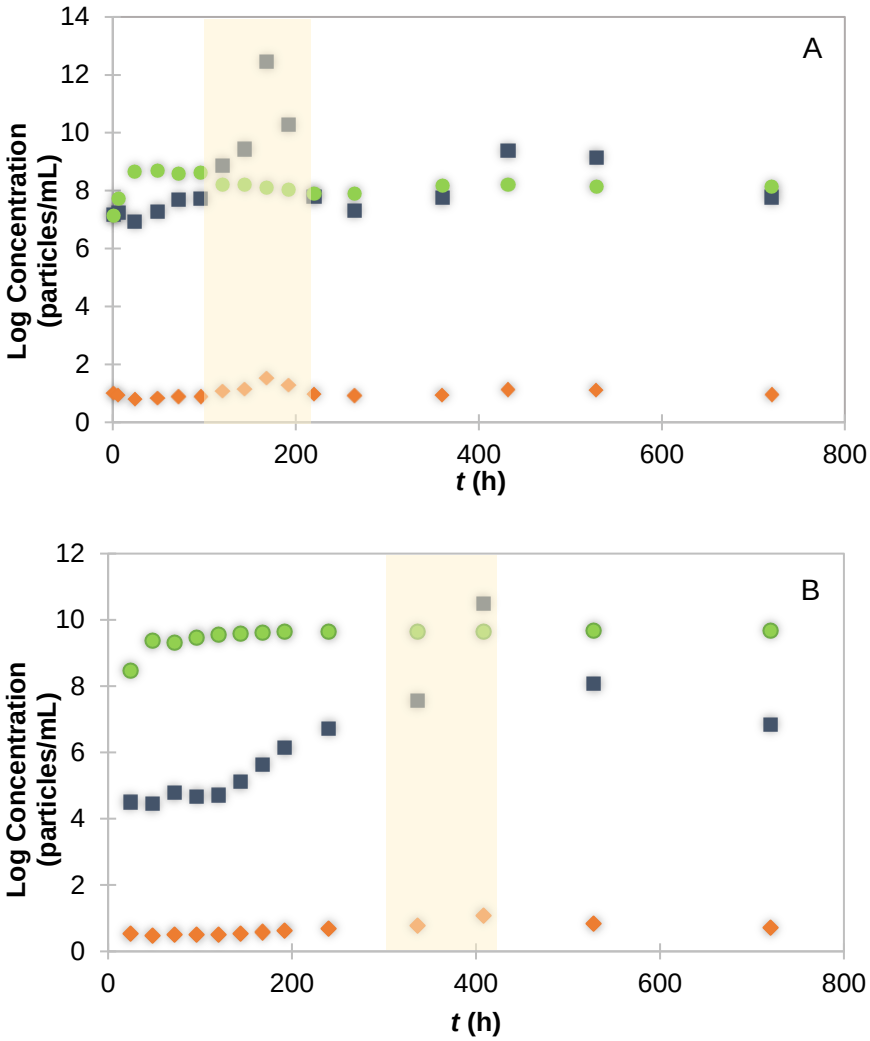


Figure IV-3: Concentration of particles in *Bacillus* sp. CCLBH 1053 in NB (A) and LAPM (B) liquid culture. (●) [Cell] (cell/mL); (■) [Spo] (CFU/mL); (◆) [Spo]/[Cell] (CFU/cell). The marked area evidences the strongest variations observed.

Despite the NB medium presents a higher concentration of spores comparatively with the cell concentration, LAPM medium shows a higher cell concentration at each time in culture batch and a lengthy spores formation process. Due to the nutrients available in the LAPM medium, the starvation and consequently, sporulation process are delayed and the cells remain longer viable and metabolically active than in the NB medium.

4.3. Antifungal compounds production

In order to detect and identify the antifungal compounds produced by *Bacillus* sp. CCLBH 1053, LC-ESI-MS analysis of the cell-free supernatant were performed along the growth. Figure IV-4 shows the LC-ESI-MS chromatogram of the culture broth and mass spectra corresponding to the LPP peaks (1031, 1045 and 1463 m/z).

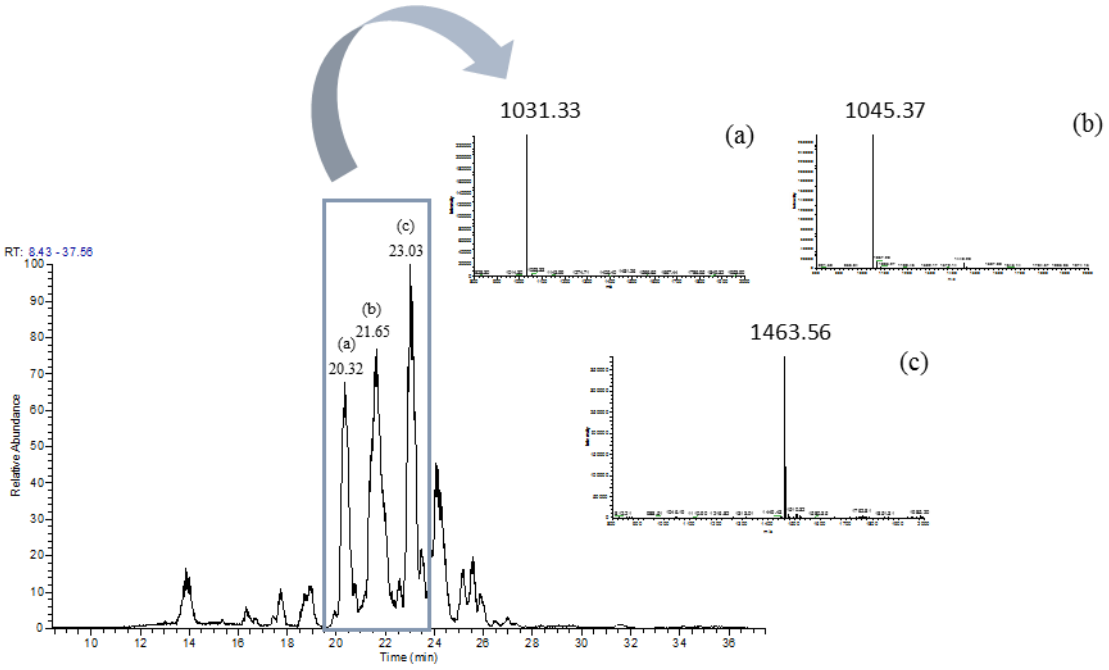


Figure IV-4: Total ion chromatogram of the cell free supernatant of *Bacillus* sp. CCLBH 1053 and mass spectra corresponding to the peaks (a) m/z 1031.33, (b) m/z 1045.37 and (c) m/z 1463.56, after 48 h of culture.

The peaks with m/z 1031 and 1045 could be assigned to iturin family and the difference of 14 Da, correspond to the loss of CH_2 (Caldeira *et al.*, 2011b), suggesting the presence of homologous molecules with different length of fatty acid chain (Figure IV-4 (a), (b)), a characteristic of lipopeptides molecules (as previous presented in section 4.2, Chapter III).

Thus, the peaks with m/z 1031 and 1045 could be assigned to lipopeptides in the iturin group and the peak with m/z 1463 suggests also the ability of these bacteria to produce lipopeptides in the fengycin group.

The evolution of the peak area of each lipopeptide group along time can give useful information for selecting the optimal time to maximise the lipopeptide yield. The profiles for the lipopeptides production along the incubation time of cultures are shown in Figure IV-5.

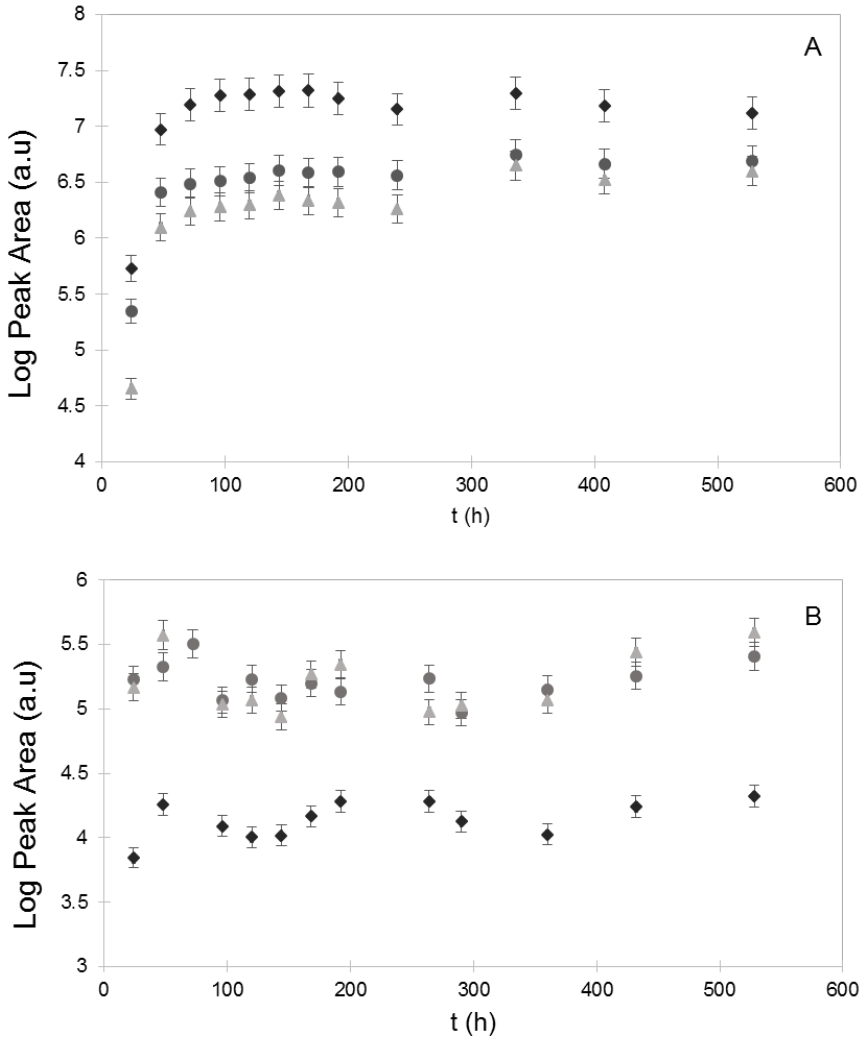


Figure IV-5: Lipopeptide production of *Bacillus* sp. CCLBH 1053 culture along culture time for NB (A) and LAPM medium (B) (◆ m/z 1463; ● m/z 1031; ▲ m/z 1045).

In the presence of NB medium, the production of lipopeptides reaches its maximum value at 168 h for the compound with m/z 1463 (fengycin group) (Figure IV-5A). For the remaining peaks (m/z 1031 and m/z 1045, iturin group) the maximum is reached later in culture time, namely at 336 h (14 days) of growth. However, the difference between the peak area profiles are clear for each LPP family. The lipopeptide corresponding to 1463 Da was produced in higher amount comparatively with the remaining compounds, with an increment of about 20% relatively to the peak 1031 and 1045 Da.

On the contrary, for the LAPM medium the molecules with 1031 and 1045 Da were produced in higher amount, comparatively to the m/z peak 1463 (Figure IV-5B) with the higher level reached early in the assay at 48 h and at 168 h. This result show that a second stage of lipopeptide production can occur after the beginning of the sporulation process at 168 h. At the end of this assay it can be also observed a new slightly increase of the three peaks area at 528 h, suggesting the presence of a continuous production of the biological active compounds along culture time. It seems that the NB medium can induced more easily the LPP with 1463 Da corresponding to the fengycin group.

Therefore, these strains show an extraordinary capacity to produce, in the two media tested, paving the way for the development of new mitigation strategies that can be used as antifungal agent, namely as new natural biocide for cultural heritage safeguard.

4.4. Cells population analysis by flow cytometry

Optical density, although indicative of microbial growth, provides few information about cell physiological state and do not take into account changes in cell size. This problem, can be overcome by the use of multi-parameter flow cytometric analysis coupled with fluorescent stains that allows the differentiation of stages far beyond the classical definition of viability (Lopes da Silva *et al.*, 2009). Complex cellular functions that are not detected by classical microbiological techniques, can be detected by flow cytometry.

In this chapter, for the physiological monitorisation of *Bacillus* sp. CCLBH 1053 cells, flow cytometric analysis was performed using a combination of two dyes, Annexin V and 7-AAD. The combined use of these dyes allow to identify and determine, by flow cytometry analysis, the concentration of live/death cells, and to distinguish cells in different apoptotic stages, early- and late-stage, as well as cells that have dead by other mechanisms.

Additionally, in order to explore the cell size as well as the spore distribution evolution in two different culture media, aliquots of *Bacillus* cell cultures were analysed, without addition of any dye, analysing the flow cytometry results in terms of forward scatter.

4.3.1. NB culture medium analysis

The percentage of early apoptotic cells (Annexin V-labelled cells) in the NB culture medium presents two periods of increase from 15% (96 h) to 25% (220 h) and from 26% (360 h) to 34% (528 h) and the apoptotic cells follow the same progress profile (Figure IV-3A). The results found, support the possibility of a correlation between the beginning of the sporulation (Figure IV-6A) and the appearance of earlier and apoptotic cells in the culture (Figure IV-3A).

According to the literature, before the loss of cell membrane integrity, the *Bacillus* cells initiate a sporulation process in response to environmental stress, leading to spore concentration increase in culture (Caldeira *et al.*, 2008). The findings observed support the existence of a possible correlation between sporulation and apoptosis.

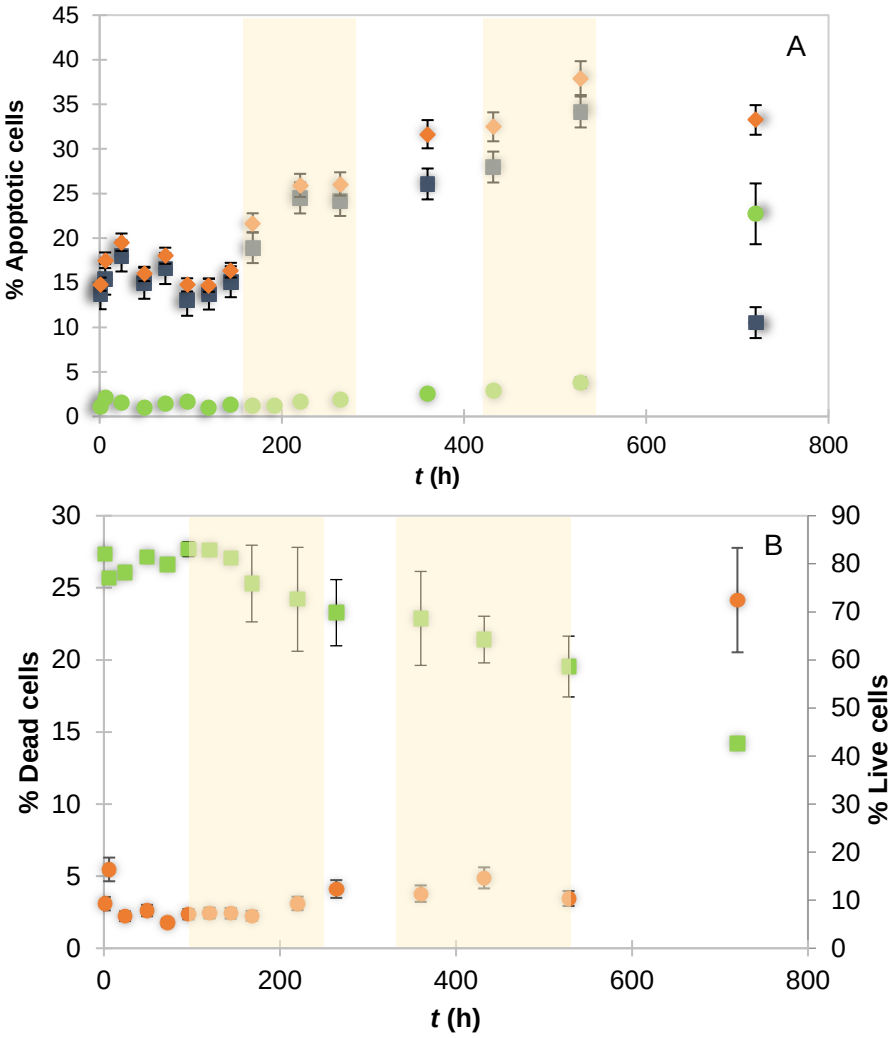


Figure IV-6: Variation of cell population distribution, of *Bacillus* sp. CCLBH 1053 growth in NB, along time assessed by staining with 7-AAD and Annexin V followed and flow cytometry analysis. A- (●) Late apoptotic cells; (■) Early apoptotic cells; (◆) Total apoptotic cells. B- (■) Live cells; (●) Dead cells. The marked area evidence the strongest variations observed.

Regarding the evolution of the cells viability, it can be observed that the percentage of dead and live cells in the culture present a comprehensibly opposite profile, as the percentage of live cells decreases along time in the culture, the percentage of dead and apoptotic cells increases (Figure IV-3B). This is due to the reduction of the available nutrients in the culture along time. In order to deepen and take on more the cell population dynamics in the culture, in addition to the cell concentration and viability data

directly given by the software, the histograms and dot-plots correspondent to the flow cytometry analysis of the samples and the corresponding blanks were also obtained. Only those correspondent to the analysis at 1, 96, 168 and 720 h of culture incubation are shown in Figure IV-7, as example, as they are the most representative. These graphs allow to follow the differences along time in terms of cells size (light scatter), by comparing the results of the controls, Figure IV-7 and IV-8 j (not of the samples, because the dyes solution addition usually contains permeabilizers that could alter the cell size). The comparison of the stained samples also allow to monitor the evolution of the viability of the cells as well as the death of the cells by different mechanisms, by apoptosis or others along time, by comparing the signals obtained for the samples along time in the yellow (apoptosis) and red detectors (Figure IV-7, a-e).

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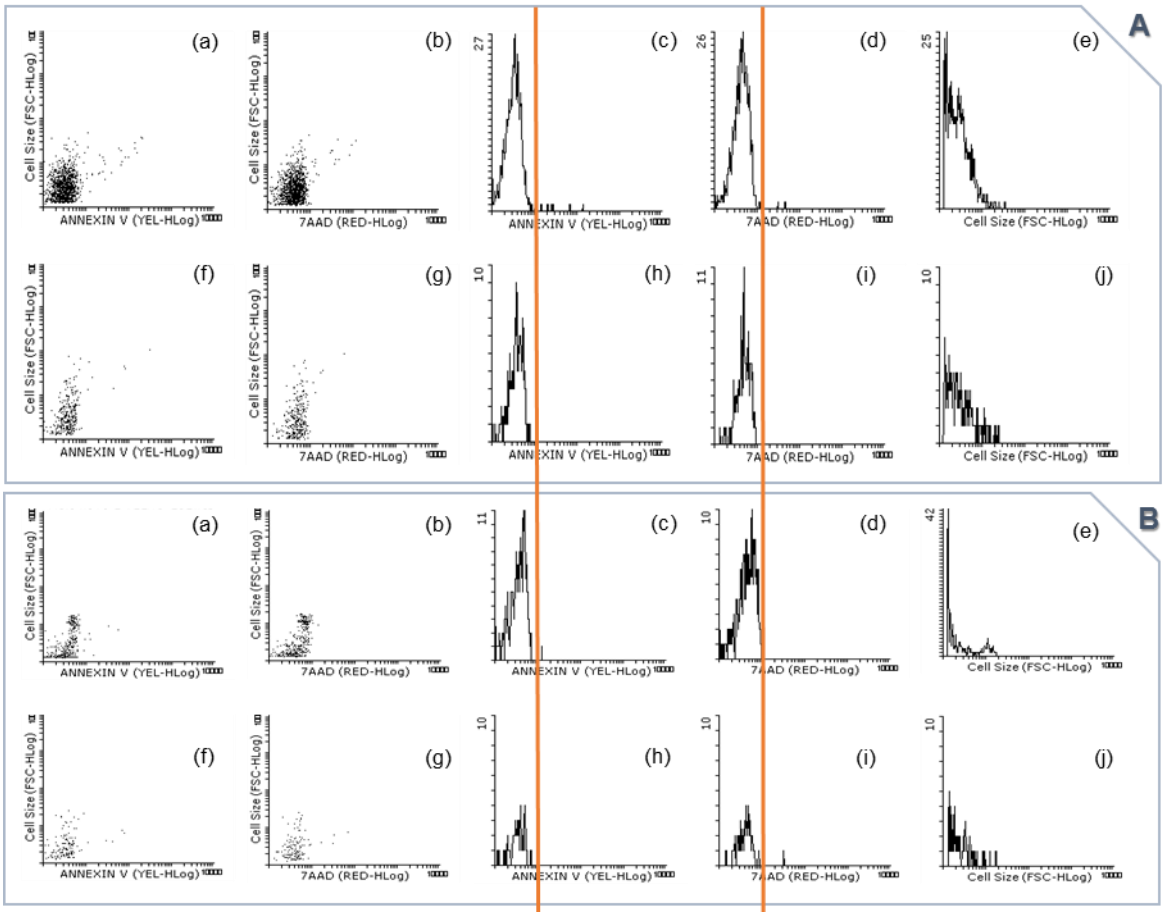


Figure IV-7: Time course of the two sub-populations of *Bacillus* sp. CCLBH 1053 growing in NB medium. A, B- Dot-plot cytometry and histograms at 1 and 96 hours of incubation, respectively. (a)-(e)- Cells stained with a mixture of Annexin V and 7-AAD dyes; (f)-(j)- No stained cells; (a), (c), (f), (h) - Cells sensed with Annexin V detector; (b), (d), (g), (i) - Cells sensed with 7-AAD detector. (e), (j) - Flow cytometer forward scatter (FSC) for cell size analysis. The orange line, represent the division between the signals obtained for the live cells and those obtained for the dead cells (dyed by apoptosis, Annexin V labelled, or died by other mechanism, 7-AAD labelled).

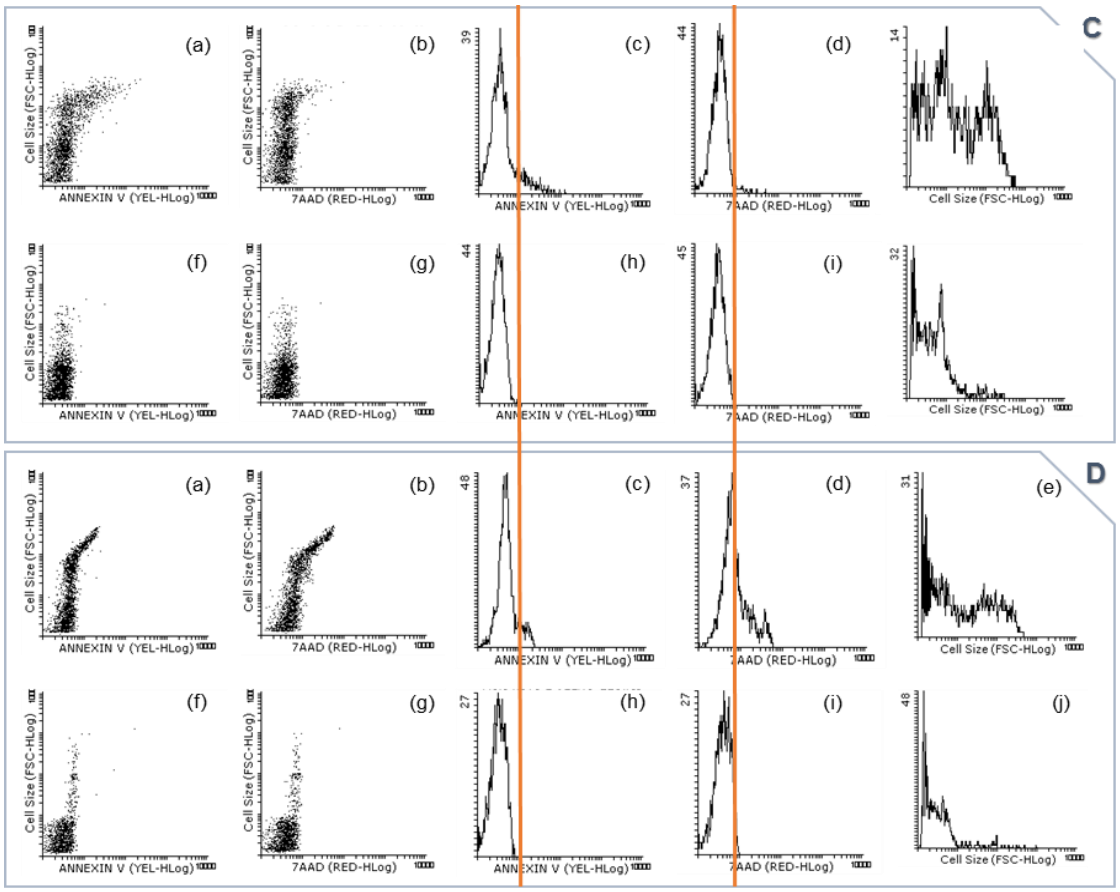


Figure IV-7 (continued): Time course of the two sub-populations of *Bacillus* sp. CCLBH 1053 growing in NB medium. C, D- Dot-plot cytometry and histograms at 168 and 720 hours of incubation, respectively. (a)-(e)- Cells stained with a mixture of Annexin V and 7-AAD dyes; (f)-(j)- No stained cells; (a), (c), (f), (h) - Cells sensed with Annexin V detector; (b), (d), (g), (i) - Cells sensed with 7-AAD detector. (e), (j) - Flow cytometer forward scatter (FSC) for cell size analysis. The orange line, represent the division between the signals obtained for the live cells and those obtained for the dead cells (dyed by apoptosis, Annexin V labelled, or died by other mechanism, 7-AAD labelled).

Differences between the blanks, without staining the cells (Figure IV-7 (f)-(j)), and the stain assays with the mixture of the two dyes were not very notorious, until the moment that start to appear apoptotic cells (t >168 h) and the signals correspond to viable cells. The cells population suffer a wide variation over time. After the 168 h of culture growth the presence of cells of larger size is evident. The emergence of signs different of that obtained for the control assay indicates, the presence of early or late

apoptotic cells (yellow detector- Annexin V) and the appearance of dead cells by non-apoptosis processes (red detector- 7-AAD).

Therefore, the Annexin V and dead cell kit allows to access the differentiation between cells which are dead by apoptotic and non-apoptotic mechanisms, enabled the *Bacillus* sp. CCLBH 1053 cell cycle study.

4.3.2. LAPM culture medium analysis

For the LAPM culture medium, the Figure IV-8 shows the results obtained by flow cytometry for this medium.

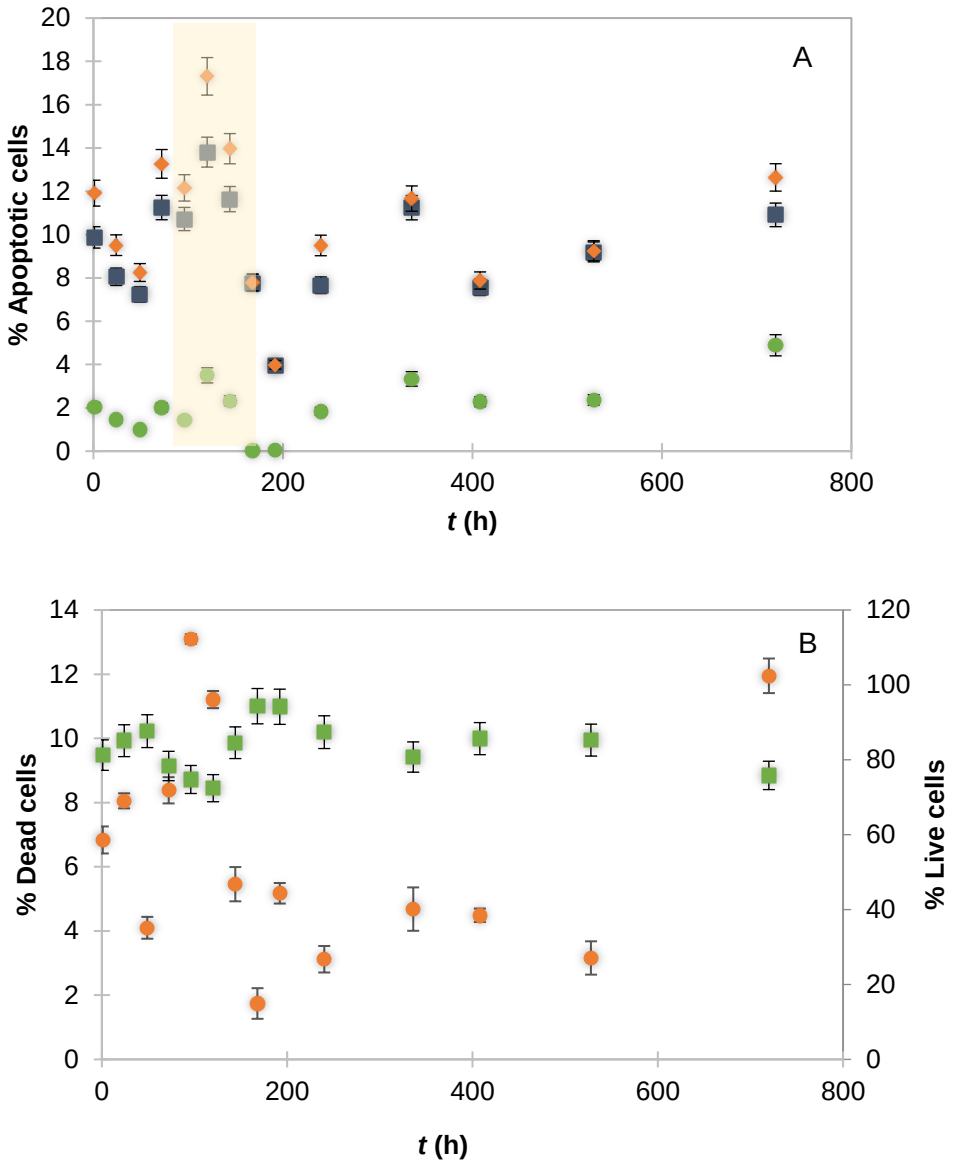


Figure IV-8: Variation of cell population distribution, of *Bacillus* sp. CCLBH 1053 growth in LAPM medium, along time assessed by staining with 7-AAD and Annexin V followed and flow cytometry analysis. A- (●) Late apoptotic cells; (■) Early apoptotic cells; (◆) Total apoptotic cells. B- (■) Live cells; (●) Dead cells.

At 120 h of incubation for the LAPM medium an increment of the apoptotic and dead cells percentage were observed (Figure IV-8A and Figure IV-8B). It can be confirmed that, the sporulation beginning corresponds to the maximal apoptotic cell concentration (Figure IV-3B). This suggests a correlation between sporulation and

apoptotic phenomena, whereas the trend found clearly depend of the nutrient availability in the culture media.

For this medium the dot-plots and histograms obtained from the cytometry analysis at 1, 120, 168 and 720 h of culture incubation are shown in Figure IV-9. For the stained samples they showed the evolution of the cells viability as well as the death of the cells by different mechanisms whereas for the controls allowed to infer about the evolution of the cell size.

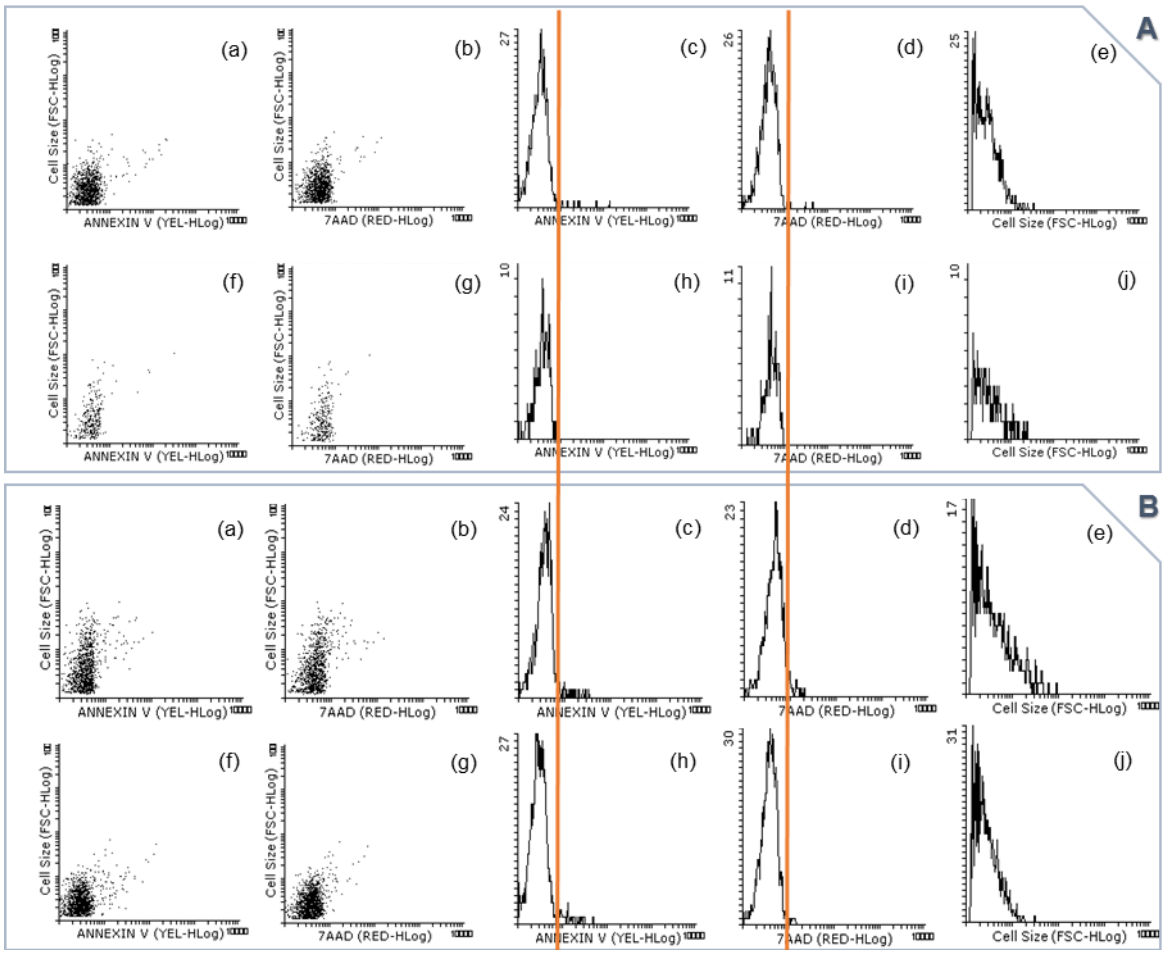


Figure IV-9: Time course of the two sub-populations of *Bacillus* sp. CCLBH 1053 growing in LAPM medium. A, B- Dot-plot cytometry and histograms at 1 and 120 hours of incubation, respectively. (a)-(e)- Cells stained with a mixture of Annexin V and 7-AAD dyes; (f)-(j)- No stained cells; (a), (c), (f), (h) - Cells sensed with Annexin V detector; (b), (d), (g), (i) - Cells sensed with 7-AAD detector. (e), (j) - Flow cytometer forward scatter (FSC) for cell size analysis. The orange line, represent the division between the signals obtained for the live cells and those obtained for the dead cells (dyed by apoptosis, Annexin V labelled, or died by other mechanism, 7-AAD labelled).

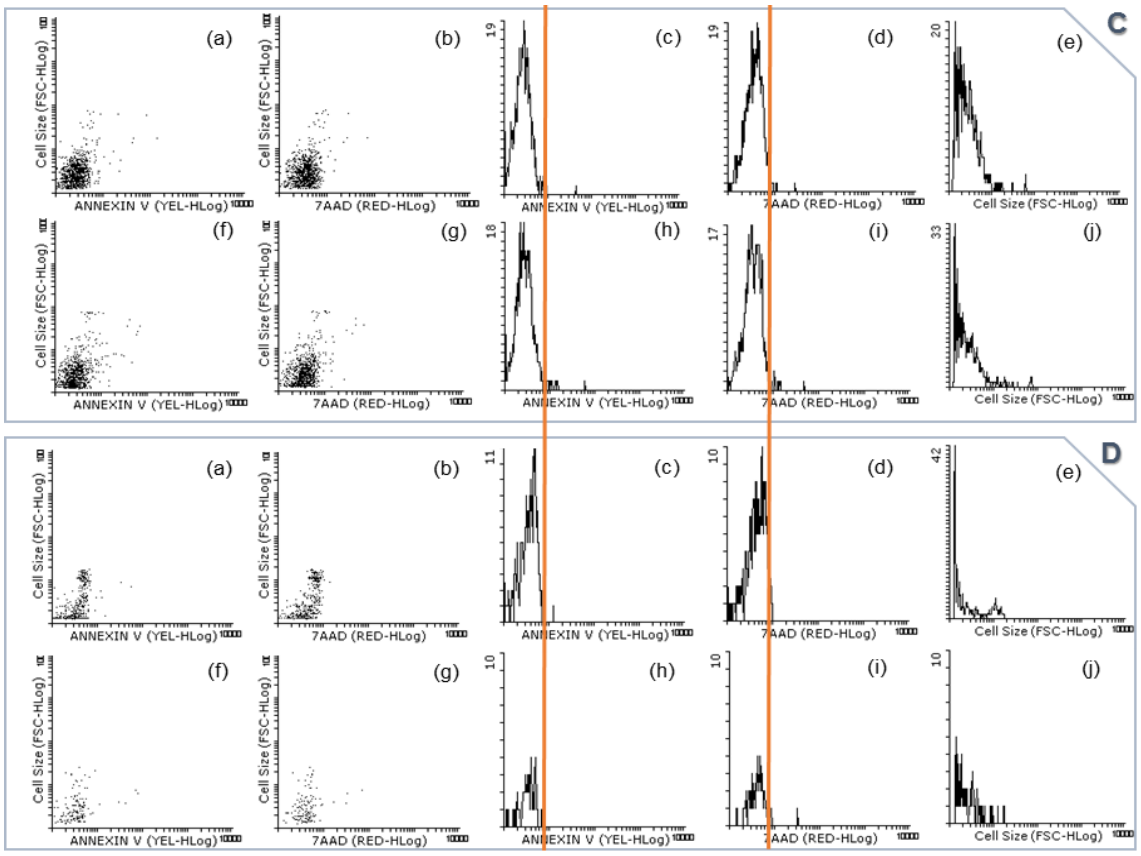


Figure IV-9 (continued): Time course of the two sub-populations of *Bacillus* sp. CCLBH 1053 growing in LAPM medium. C, D- Dot-plot cytometry and histograms at 168h and 720 hours of incubation, respectively. (a)-(e)- Cells stained with a mixture of Annexin V and 7-AAD dyes; (f)-(j)- No stained cells; (a), (c), (f), (h) - Cells sensed with Annexin V detector; (b), (d), (g), (i) - Cells sensed with 7-AAD detector. (e), (j) - Flow cytometer forward scatter (FSC) for cell size analysis. The orange line, represent the division between the signals obtained for the live cells and those obtained for the dead cells (dyed by apoptosis, Annexin V labelled, or died by other mechanism, 7-AAD labelled).

The flow cytometry analysis of cells in LAPM medium, until the moment that start to appear slightly apoptotic cells (t >120 h), did not show marked differences between the blank, without staining the cells (Figure IV-9 (a)-(e)), and the signs in the red (7-AAD) and yellow detector (Annexin V). However, apoptotic cells only notorious emerge monitoring time (t = 720 h) with the cells remaining viable for a longer period of time, confirmed the previous results and the effect of a more nutrient culture medium in the physiology of *Bacillus* cells. Moreover, the light scatter parameters show an increase in the number of bigger cells in time course.

Finally, and comparing the two culture media, a much higher percentage of live cells are present in the LAPM (around 90%) than in the NB medium (80 to 40%) independently of the culture incubation time. The opposite trend is observed for the total apoptotic cells where the slight increase remain at 25% in LAPM, when NB in medium present more than 40% for the same cells. This allows to affirm that the cell population dynamic is strongly affected by the composition of the culture medium.

These results confirmed, what was found in previous studies, that the influence of the nitrogen source concentration in the viability of *Bacillus* cells in the culture broth became more evident that the time of incubation (Rahman *et al.*, 2006), although the association of these two factors is particularly relevant in the spores formation and the expression of the antifungal activity (Caldeira *et al.*, 2008).

4.5. Spore germination study

In order to complete the comprehensive study of sporulation of *Bacillus* sp. CCLBH 1053, a spores germination assay was performed in which the NB culture received, after 72 h of growth, a nutrient supplementation with fresh Peptone in the NBPS assay and a Peptone supplementation with an additional heat-activation step in the NBPSHA assay.

The evolution profile of cells and spores are shown in the Figure IV-10.

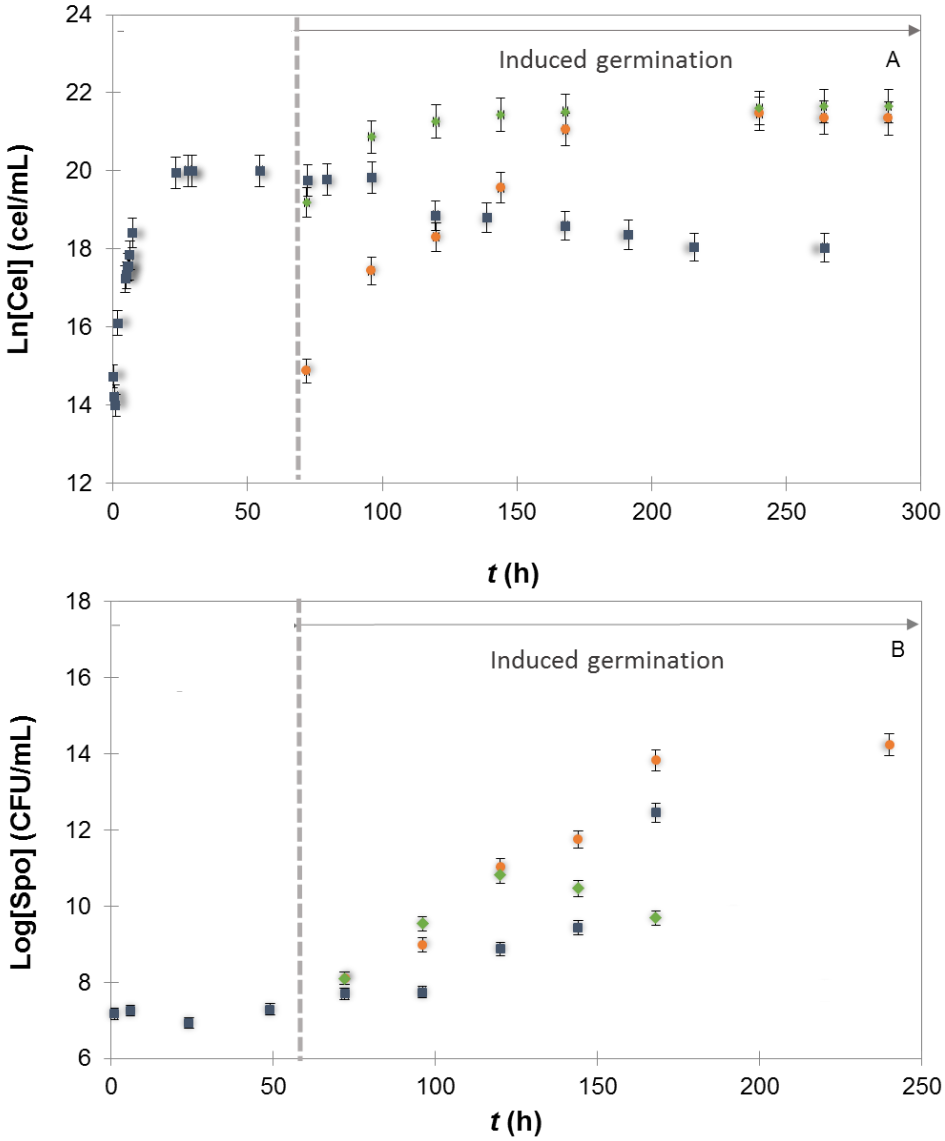


Figure IV-10: Time course profile of *Bacillus* cells (A) and spores formation (B), before and after the peptone supplementation and heat activation assays. (■) Normal growth in NB culture medium; (●) NBPSHA assay; (◆) NBPS assay.

In the instant that peptone supplementation was performed, the cells are set in the stationary phase of the culture growth (Figure IV-10A). After the heat shock of 77ºC for 12 min (NBPSHA assay), the concentration of cells has decreased. However, after an adaptation period, the concentration of cells in the culture broth rise again. Thus, it can be pointed out that the nutrients addition to the culture broth to cells in the stationary

phase, induces *Bacillus* cells proliferation reaching a new maximum concentration for both NBPS and NBPSHA assays at 240 h of culture growth (about 2.55 x 10^9 cel/mL).

However, the same profile was not observe in the case of spores formation (Figure IV-10B).

When the peptone supplementation was completed at the 72 h of incubation, the production of spores had not initiated. After 24 h of culture peptone feeding moment (NBPS and NBPSHA assay), the spores initiated a gradual progress up to 120 h of culture total time course profile, with a concentration of 3.08 x 10^9 CFU/mL. However, at this time the spores in the NBPS assay began to decrease, instead NBPSHA assay remains to surge, reaching its maximum value at 240 h of total incubation (1.77 x10^{14} CFU/mL).

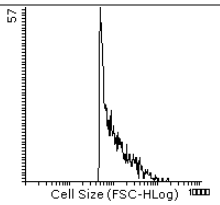
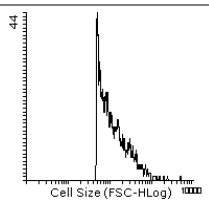
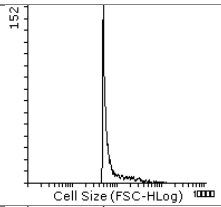
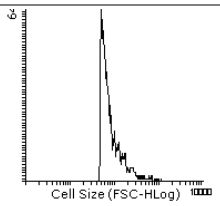
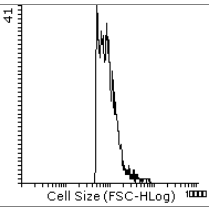
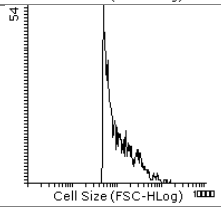
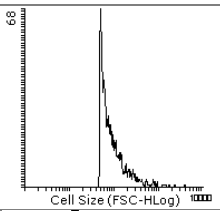
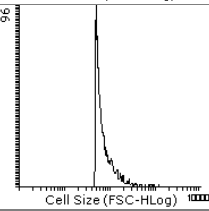
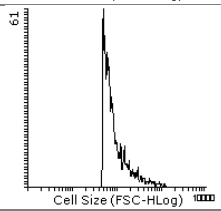
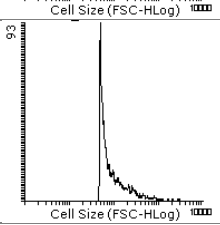
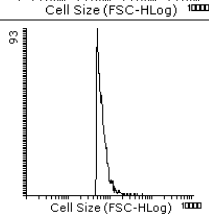
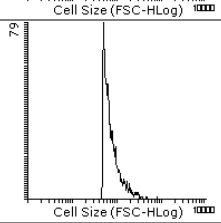
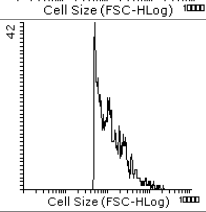
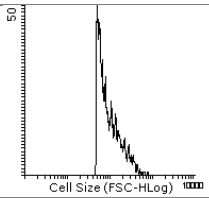
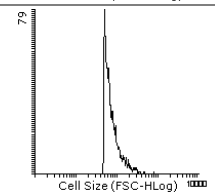
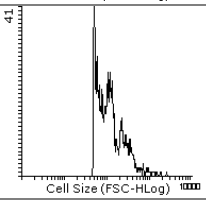
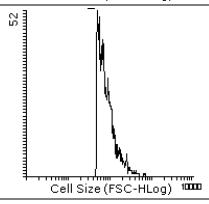
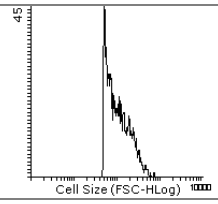
The thermal shock became, in this way, more effective for spore germination. In fact, when the culture was supplemented with Peptone followed by a sub lethal heat shock of 77ºC to activate the spores, the cells become metabolically active in the culture.

Furthermore, and as it is pointed out by the results shown above, the maximum cell concentrations for both assays were almost the same, indicating that peptone supplementation with heat activation, only influenced the germination of spores rather than altering the final concentration of total cells.

Rahman *et al.* (2006) revealed that a supplementation with fresh peptone and an heat-activation of an old culture medium led to a induced spores germination and another round of production of the lipopeptide antibiotic iturin A (Rahman *et al.*, 2006). The present study reveal that peptone supplementation induces spore germination and the heat shock promote an initial decrease on cells concentration, supporting and complementing the previous reported study.

For a completed induced spores germination analysis, the presence of *Bacillus* spores and the different cell subpopulations were directly monitored, without addition of any dye, by flow cytometry according to the light scatter parameters (Table IV-1).

Table IV-1: Flow cytometer forward scatter (FSC), fluorescence histograms of *Bacillus* sp. CCLBH 1053 culture in NB and of two different sporulating assays in the same medium with Peptone Supplementation (NBPS) only, or followed by heat shock (NBPSHA) in different time of incubation.

t_{total} (h)	$t_{after\ treatment}$ (h)	NB	NBPS	NBPSHA
72	0			
96	24			
120	48			
168	96			
240	168			
336	264			

The cells in the NB medium after 72 h of growth possess different sizes, and from 72 h to 168 h, in accordance with the results obtained in the sporulation assay (Figure IV-3A), the population of smaller cells (spores) increases reaching a maximal concentration at 168 h (at this total time of incubation, the peak correspondent to the spores is higher than for the other times of incubation analysed). In accordance with the

FSC histogram, the spore population represent the majority over the other type of cells at this time. After longer periods of incubation it can be observed that the spore population decreases at the same time than the population of cells of greater size increases (dead and apoptotic cells).

However, whereas immediately after treatment (t=0 h) the NB and NBPS population distributions are similar. Over time in the NBPS assay the smaller size cells (spores) take a preponderant role into the whole population. At 120 and 168 h the cells distribution is centred mainly in the spore population, representing, in this way the majority of the cells. After that the histograms showed once more the presence of largest size particles, but not with a representative population of apoptotic or dead cells, even at 336 h of incubation. This is in accordance with the cell and spores concentrations profiles obtained for the NBPS assay (Figure IV-10).

For the NBPSHA assay, the light scatter parameters analysis shows at 72 h of incubation, just after treatment, a peak corresponding to smaller cells, spores. By comparing the results obtained, at the same time of incubation, for NB and NBPS treatment, it can be concluded that the heat shock produces an immediate decrease of the largest size cells (that can be attributed to their lysis). After the heat shock a progressive appearance of bigger cells in time course is observed, suggesting the increase of germination process. However, at 168 and 264 h, once again, the spore population represents the majority over the other type of cells.

This results confirm once more the previous ones and the advantages in performed a nutrient addition in the old production medium that can overcome successfully by heat activation.

Despite flow cytometry with the light scatter parameters only explore the size of the cells, it gives us a worthy additional information about the spores and cells distribution and germination process, becoming a useful tool for sporulation monitorisation of *Bacillus* strains.

Once the spore germination investigation was completed, the influence of NBPS or NBPSHA treatment into the production of bioactive compounds were study in order to explore if they could represent good alternatives to enhance their production.

The relative quantification of bioactive compounds produce by *Bacillus* sp. CCLBH 1053 was followed by LC–ESI-MS in positive mode after the peptone supplementation or the combined treatment with heat activation. The production of lipopeptide with m/z 1031, 1045 and 1463 peaks, corresponding to iturin and fengycin lipopeptides family was determined during 7 days of incubation for NBPS and NBPSHA assays (Figure IV-11).

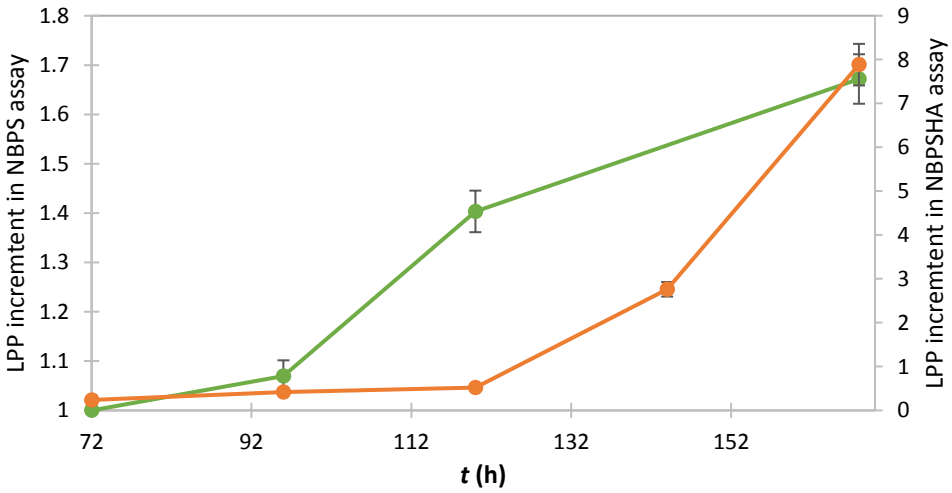


Figure IV-11: Lipopeptide production increment for NBPS assay and NBPSHA assay. The relative percentage of increase was calculated considered as 100% the area of peak corresponding to 0 h of incubation time after treatment. (—●—) NBPSHA; (—●—) NBPS assay.

In the NBPS assay the LPP productivity increases 1.6 times when compared to that obtained in NB medium. However, in the NBPSHA assay the increment was significantly higher than for the previous assay, the heat shocking promotes an increment production 8 times higher relatively to normal growth in NB medium. In fact, the activation by nitrogen supplementation and heat shock at 77ºC generated metabolically active cells with higher capacity to produce LPP.

Therefore, a supplementation with a new nitrogen source and heat activation can be apply for increasing lipopeptides production in the later phases of *Bacillus* sp. CCLBH 1053. These results confirmed previous studies that described that only nutrient addition cannot exclusively improve the production of iturin A in the old culture medium, but also can overcome successfully by spores activation (Rahman *et al.*, 2006; Caldeira *et al.*, 2008).

5. Conclusions

Flow cytometry coupled with specific fluorescent dyes were used to physiological monitorisation of *Bacillus* sp. CCLBH 1053 culture. The use of this technique allowed both detection and quantification of sporulation inside a culture, and distinguished cells that differed in viability. The use of such a rapid, real time analysis of microbial cells is enhancing our knowledge of how different physiological sub-populations develop with time (population dynamics) throughout a bio-process, demonstrating the inherent complex heterogeneity of microbial cells. The capability of the fluorescent staining methods in order to identify a number of dynamic sub-populations with different functional characteristics suggests a possible use for optimisation of bioprocess performance. Also, the flow cytometry was used to explore the bacteria cells size by forward scatter in order to accomplish the spore distribution and complement the study of cell cycle.

Furthermore, metabolically active cells derived from induced germination of the *Bacillus* sp. CCLBH 1053 spores were responsible for re-production the iturin and fengycin lipopeptides after their first metabolic stage. The analysis of the natural behaviour of CCLBH 1053 cells related to it sporulation capacity, establish the relation between the sporulation process and the bioactive compounds production.

A supplementation with a nitrogen source and a heat activation can be apply on the lipopeptides production in the latest phases of *Bacillus* sp. CCLBH 1053 cultures,

constituting a new tool for metabolically activation of spores in the production medium, with significant potentiality in the production improvement of these natural metabolites to be use in the production of novel green biocides for Cultural Heritage.

CHAPTER V

Toxicological assessment and antifungal efficiency of the bioactive compounds

Some results of this chapter were published in the following scientific publications:

Silva M., Salvador C., Candeias M. F., Teixeira D., Candeias A., Caldeira A.T. (2016). Toxicological Assessment of Novel Green Biocides for Cultural Heritage. Int J Conserv Sci 7, SI1, 2016: 265-27

Silva M., Rosado T., Teixeira D., Candeias A., Caldeira A. T. (2015). Production of green biocides for cultural heritage- novel biotechnological solutions. Int J Conserv Sci 6 (SI): 519-530

1. Overview

The need of safe and effective antifungal agents increases in parallel with the expanding number of the studies that blame fungal communities for degradation and deterioration of built culture heritage.

The chemical biocides have been usually used, until now, to mitigate this problem. However, in the past decade most of the effective biocides have been banned due to the environmental and health hazards associated with them. It is therefore imperative to find antifungal compounds and new solutions that are non-toxic to mammalian cells and to the environment itself.

This chapter presents a toxicological evaluation, using two different biological models, *Artemia salina* and S*wiss* mice, of the new bioactive compounds produced, by the selected strains of *Bacillus* sp.. Also, simulation studies, using fungal contaminated mortar slabs and real mural painting samples, were performed in the presence of the new compounds in order to simulate and explore the real life efficiency of the compounds and their possible influence on the structure and colour of real mural painting samples.

The compounds produced by *Bacillus* sp. besides the great potential to suppress biodeteriogenic fungi growth from historical artworks, proven once more in simulation assays, present no toxicity against living organisms (namely mammals) constituting an efficient and green safe promising alternative for biodegradation/biodeterioration treatment of artworks.

2. Introduction

The damage of buildings and monuments by biological contamination is a cause of serious concern. Biological growth is a potential cause of long term decay, associated to physical and chemical damage of building materials (Stein, 2005). The prevention of microorganism colonisation in Cultural Heritage assets as well as the development of appropriate treatment measures for contaminated objects is a challenge for restorers, museum curators and architects (Sterflinger, 2010; López-Miras *et al*., 2013). Conservative interventions using direct and indirect methodologies have been applied in order to stop or slow-down the biodeterioration process (Gaylarde *et al.*, 2011). The methodologies and products must be chosen taking in mind the substrate conditions and the species to be treated, trying to produce low environmental impact as well as to avoid negative interferences with the materials (De los Ríos *et al.*, 2012). This has implications for the techniques of cleaning and conservation of the objects but also consequences for the occupational safety and health of restorers.

To eliminate the biodeteriogens of cultural objects, many organic and inorganic compounds have been used, as biocide agents (Richardson, 1988; Fonseca *et al.*, 2010; Roig *et al.*, 2013). However, in the past decade many of the most effective biocides have been banned due to the environmental and health hazards associated to them or to their use (Young *et al.*, 2008).

In some reported cases, several products have been used in order to eliminate the biodeteriogenic organisms that thrive on stone monuments or rock sites (Urzì and De Leo, 2007; Bastian *et al.*, 2009; Scheerer *et al.*, 2009). Until now the most common biocides used were disinfectants that destroy vegetative forms but that were not always effective against survival resistant or quiescent phase structures, and pesticides that eradicate undesirable biological growth with a specific toxicity for the species to be eliminated (Tiano, 2002; Nugari and Salvadori, 2003). 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 sodium and calcium hypochlorite to more complex organic ones such as the Quaternary Ammonium Compounds, *i.e.* Preventol R50 or Neo-Desogen (Hegstad *et al.*, 2010; Buffet-Bataillon *et al.*, 2012). However, these chemical biocides act through toxic mechanisms 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 (Ashraf *et al.*, 2014). Organotin compounds biocides possess, for instance, a strictly specific mode of action. TBTO urea derivatives (Diuron, Karmex), for example, block the photosynthetic process (Tiano, 2002).

Any biocide intended for use on historic monuments must be not only effective against biological growth but at the same time need to cause no damage to the material either by direct action or by leaving deposits which may result in successive damage (Scheerer *et al.*, 2009). Furthermore, it must be underlined the necessity of testing these products under analogous “real cases” conditions as the interactions among the substrates, biocides and organisms can give a more realistic indication of how a biocide will perform *in situ* (Allsopp and Allsopp, 1983).

Therefore, developing proper 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 cultural heritage treatment and rehabilitation due to its capacity to produce a great diversity of secondary metabolites with biological activity, as it has been previously described. In fact, strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been referred to produce antifungal lipopeptides (Leifert *et al.*, 1995; Caldeira *et al.*, 2008; Das *et al.*, 2008a) such as surfactin, fengycin and iturin. These amphiphilic membrane-active biosurfactants present potential antagonistic activities against many fungal pathogens (Moyne *et al.*, 2004). Due to their low molecular weight, these 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 interfered on the microorganism's adherence and detachment from surfaces (Stein, 2005). 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 (Maget-Dana and Peypoux, 1994; De Lucca and Walsh, 1999; Yao *et al.*, 2003). 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 chapter was envisaged to evaluate the safe toxicological properties and real life efficiency of the new bioactive compounds produced.

For the toxicological assessment two different biological models, brine shrimp (*Artemia salina)* and Swiss mice (*Mus Muculus*) were used. While the first model was used as a standard test for determination of lethal concentrations (LC_{50}), the second one, worked as a mammal cell model to test acute toxicity of the biocompounds produced for the determination of lethal dose (LD_{50}), according to Organization for Economic Co-operation and Development (OECD) guidelines.

Additionally, simulation studies using fungal contaminated mortar slabs and mural painting real samples, were performed in the presence of the new compounds in order to simulate and explore the real life efficiency of the compounds and their possible influence on the structure of real mural painting samples. For this intent, simulated and real mortars (from mural painting), and the effect of the new compounds was evaluated periodically during six months.

3. Materials and Methods

3.1. Microorganisms and bioactive compounds production

The strains of *Bacillus* sp. CCLBH 1051, CCLBH 1052 and CCLBH 1053 were maintained on Nutrient Agar (HIMEDIA) slants and stored at 4ºC. The bioactive compounds were produced as previously described in Chapter III (Section 3.2.).

The supernatant, Culture Broth (CB) was maintained at -20ºC for further analysis and will be designated in this chapter by CB1, CB2 and CB3, corresponding to *Bacillus* sp. CCLBH 1051, *Bacillus* sp. CCLBH 1052 and *Bacillus* sp. CCLBH 1053, respectively.

3.2. Acute toxicity assessment

3.2.1. Toxicity in *Artemia salina*

The toxicity of the lyophilised 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 the *Artemia salina* test kit (Artoxkit MTM, Microbiotest).

To evaluate the toxicity of the samples, 900 µL of saline medium (Annex F, Table F-1) and 100 µL of the samples were placed in each well of the 24-plate. In each well 10 Nauplius (I / II larval stage), previously rehydrated and developed from the cysts, were placed and incubated in a saline medium, in the absence of sun light, at 24ºC, during 24 h. Subsequently, the number of fixed microcrustaceans in each well were counted with the help of Leica M205C stereozoom microscope (Hartl and Humpf, 2000).

All the biocides were tested in a range of concentrations of 0.1-1000 µg/mL in order to stablish lethal concentrations (LC_{50} - lethal concentration 50%). A solution of sea

water (Artoxkit MTM, Microbiotest) and potassium dichromate ($K_2Cr_2O_7$, 100 mg/mL) were used as negative and positive controls, respectively (Salvador *et al*., 2012).

To determine the value of lethal concentration to 50% of the population, $K_2Cr_2O_7$ solutions from 10 to 100 mg/mL were used as reference (Annexe F, Figure F-1). For each compound tested three replicates were used.

The LC_{50} value for potassium dichromate was determined using the dose-response correlation by the following equation:

$$y = A_1 \frac{A_2 - A_1}{1 + 10^{(\log_x 0 - x)p}}$$

where A_2 and A_1 are respectively the maximum and the minimum response of *Artemia salina* with a control sample, *p* is the dose-response curve slope parameter, x is the concentration used and log x_0 is the concentration that corresponds to 50% toxicity. This non-linear model was fitted using Origin Pro 8.5.1 (OriginLab® Corporation, 2013).

3.2.2. Animals

The acute toxicity assay was performed using *Swiss* mice (*Mus musculus*). The lethal dose 50 (LD_{50}) was evaluated through the up and down protocol according to the guidelines of the OECD 425.

Two-month-old male albine *Swiss* mice with, (40 ± 5 g) were used. The animals were randomly divided into five groups, each one with three animals.

The animals were kept under controlled temperature (23 ± 1°C) with 12 h light - 12 h dark and fasted 24 h before testing, with water *ad libitum*. All the experimental animals' procedures were followed 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).

3.2.3. Acute toxicity in *Swiss* mice

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 1000 mg/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 (Figure V-1).

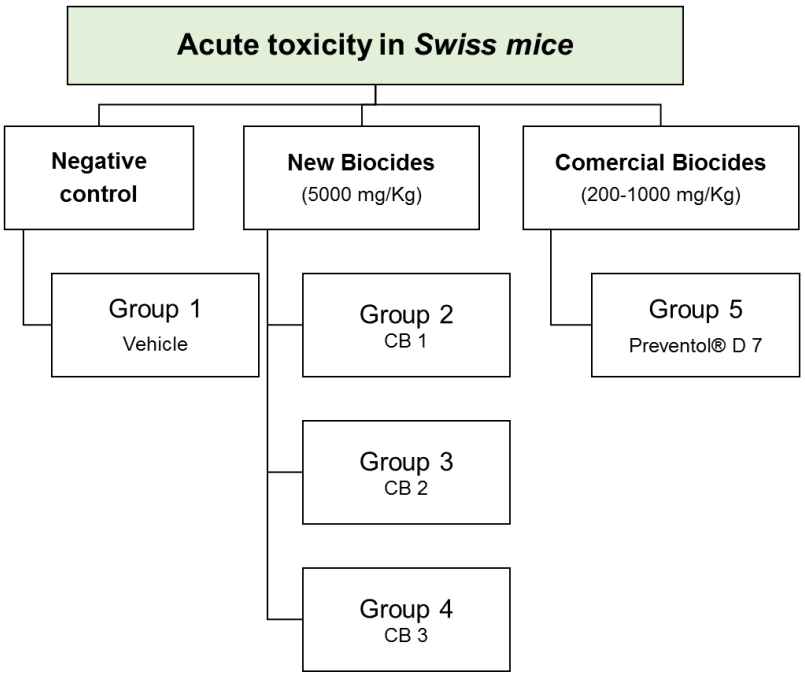


Figure V-1: Schematic representation of the acute toxicity assay on *Swiss* mice.

Additionally, a pharmacological screening was performed in order to observe the behaviour and activity of the animals at 0, 1, 2, 4, 6 and 24 h 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) (Figure V-2).

The animals were kept under observation for 15 days (OECD, 2001) in the conditions described above (section 3.2.2.).

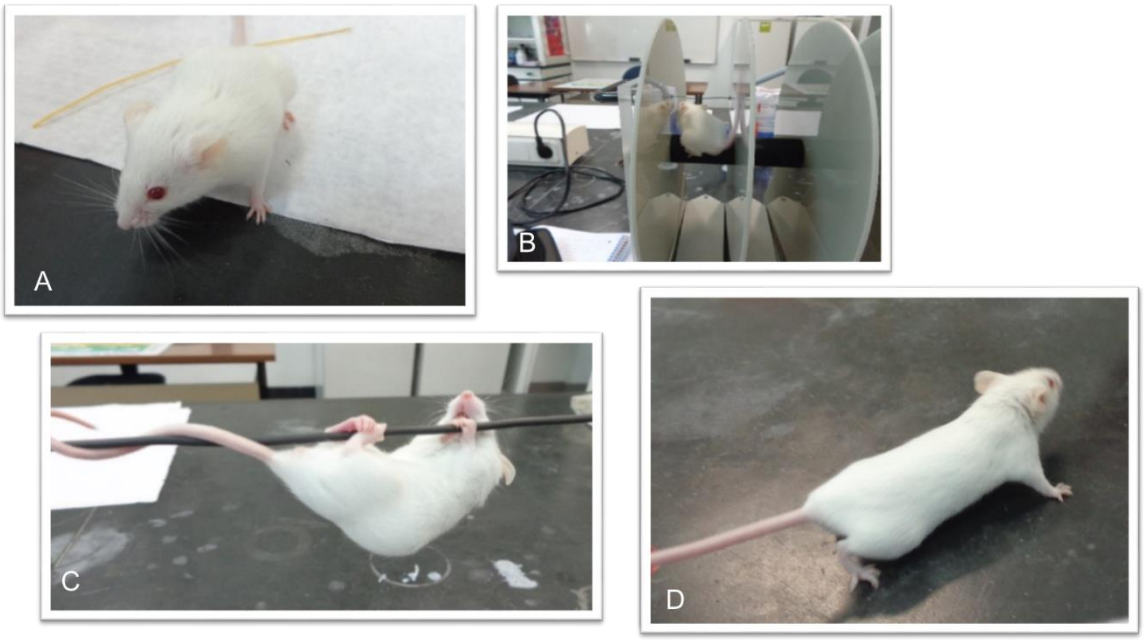


Figure V-2: *Swiss* mice toxicity assay. A- Pineal reflexes tests, B- Motor activity teste, C- Traction tests and D- Behaviour observation in toxicological evaluation assay.

3.2.4. Statistical analyses

The results of the acute toxicity assays with *Artemia salina* and *Swiss* mice, after treatment with the bioactive compounds produced by *Bacillus* strains, were evaluated statistically using the SPSS® 22.0 software for Windows Copyright©, Microsoft Corporation (Annexe G). Descriptive parameters and One-way ANOVA were used in order to determine statistically significant differences at the 95% confidence level ($p<0.05$). The population variances homogeneity was confirmed by Levene test, being

considered significant values whose probability of occurrence is greater than 95% ($p < 0.05$).

3.3. Simulation assays with mortar samples

3.3.1. *In vitro* assay using mortar slabs

To evaluate real efficiency and influence of the new compounds on the activity of biodeteriogenic microorganisms, a combined strategy using the bioactive molecules (Section 3.2) under *in situ* controlled conditions was performed.

Mortar slabs of 3 x 6 cm were manufactured in the laboratory using Vitruvius formulation (Cardoso *et al.*, 2014) and cured for two weeks. Mortars were composed by two mixed binders, aerial lime and siliceous sand (1:3).

After sterilisation, two different assays were performed in parallel as shown in the methodological scheme (Figure V-3).

Mortars Slabs (MS1) were inoculated with a mixture composed by 1 mL of *Cladosporium* sp. 10^5 CFU/mL spore suspension (prepared as described previously in Chapter III, Section 3.3.1.) and 1 mL of malt extract and 500 µL of the cell-free supernatant produced by *Bacillus* sp. CCLBH 1051, CCLBH 1052 and CCLBH 1053 (CB 1, CB 2 and CB 3). A control assay was also performed in the absence of the new compounds (avoiding the addition of supernatant). The slabs were incubated at 25ºC, monitored periodically and documented using a digital camera (Sony DSC-W730).

After 2 months of incubation all the slabs were air-dried, coated with gold and examined with a HITACHI 3700N (Tokyo, Japan) variable pressure Scanning Electron Microscope (VP-SEM), coupled with a Bruker XFlash 5010 Energy Dispersive X-ray (EDS) spectrometer (Berlin, Germany) with an accelerating voltage of 18-20 kV, to allow visualisation of the surface “biofilm” and elemental composition (point analysis and two-dimensional mapping).

3.3.2. Real mortars under the presence of the new biocides

Real mortar samples from a mural painting was monitored, after the application of the novel biocide compounds in order to evaluate the possible structural or superficial modification.

Two series of assays were performed using real mortars obtained from detached fragments (about 4 cm^2) of a mural painting (*Santo Aleixo* Church, Montemor-o-Novo, Portugal) as described in Figure V-3.

The first series of assays were prepared impregnating the real samples with 200 µL of the extracted new compounds (3 mg/mL) - RM1 series (Figure V-3). For this assays, a portion of the compounds produced in liquid cultures were isolated from culture broth by adding 3 M HCl to a final pH of 2.0. The precipitates were collected and extracted with methanol. The methanol soluble compounds were vacuum-dried and recovered for further analysis.

The second series of mortar assays were prepared impregnating the surface of the painted mortar with 500 µL of CB 3 (*Bacillus* sp. CCLBH 1053 cultures) - RM2 (Figure V-3).

During six months the possible surface alteration of the painted mortars was photographically documented, using a digital camera (Sony DSC-W730). At the end of the assay, the possible modifications were recorded with Leica M205C stereozoom microscope, acquiring the images with a Leica DFC290HD camera. Colour evaluation was also performed with a Datacolor CheckPlus II spectrophotometer (DataColor, NJ). The results obtained in the CIE L*a*b* chromatic space defined by the CIE in 1976, were 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 axes (0–100). The diffuse reflection spectral curve of the pigments in the visible range (380–740 nm) was also measured.

The results of the CIE L*a*b* chromatic space were evaluated statistically (Annexe G), using the SPSS® 22.0 software for Windows Copyright©, Microsoft Corporation, by descriptive parameters and by One-way ANOVA in order to determine statistically significant differences at the 95% confidence level ($p<0.05$). The population variances homogeneity was confirmed by Levene test and multiple average comparisons were evaluated by Tukey test, being considered significant values those whose probability of occurrence is greater than 95% ($p <0.05$).

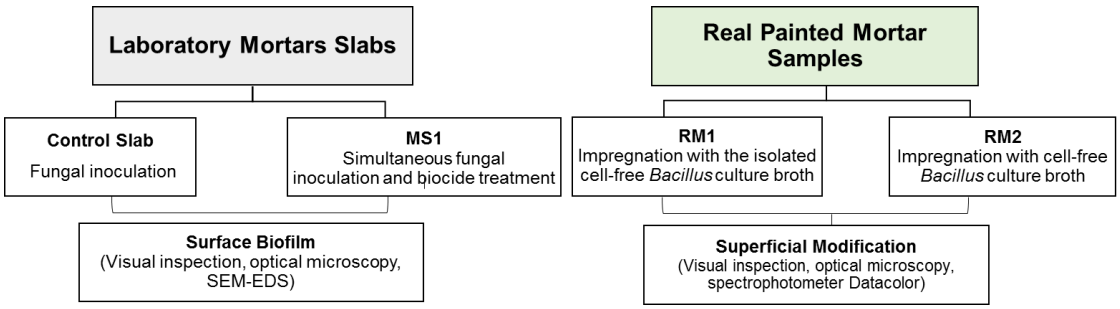


Figure V-3: Methodological scheme of simulation *in vitro* assays using mortar slabs and real samples under the presence of bioactive compounds.

4. Results and Discussion

Increasing attention is paid in the field of Cultural Heritage conservation for sustainability, inviting researchers to look for innovation of traditional working materials and methods, in order to reduce or avoid possible damages to health and environment. In fact, conservators-restorers or similar staff (i.e. visual artists, museum workers) are exposed to a series of hazards (i.e. contact with biocides, solvents, organic dust including endotoxins, moulds and mites) but few data are available regarding their health (Caminitti *et al.,* 2016). This enhances the problem of the inadequate consideration of health and safety risks.

The criteria for a green biocide were established according to the principles of green chemistry. A biocide must fulfil all of the requirements before it can be declared safe for living beings and the environment, and, to be considered green.

In this last chapter, the principal intent was to perform a toxicological assay using different biological models for the evaluation of the most effective and efficient bioactive compound produced by *Bacillus* cultures from those established in previous chapters. The natural compounds were also compared with commercial chemical biocides (Preventol® D 7, NEW DES® and Panacide®) commonly used by conservators/restorers in built heritage. This have allowed to clarify and prove the absence of toxicity against superior beings of these new products but also to confirm their green safe characteristic.

In parallel, the most effective biocompounds were tested in a simulation assay using mortar slabs with evident signs of fungal proliferation and also in real samples, using mural painting fragments. These assays allow to establish once and for all, the potential of the natural compounds produced by strains of *Bacillus* sp. for novel environmental safe biocide for Cultural heritage.

4.1. Toxicological evaluation

The toxicity of the bioactive compounds that are presente in the lyophilised 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) (OECD, 2001).

4.1.1. Toxicity in *Artemia salina*

The toxicity of the bioactive compounds produced were firstly evaluated on *Artemia salina*.

The lyophilised supernatants of the *Bacillus* CBs show a low toxicity on *Artemia salina* (less than 3,5%) at a concentration of 1000 µg/mL. Seawater, used as negative control, caused no mortality whereas, potassium dichromate, used as positive control, cause 95.5% of mortality at a concentration of 100 mg/mL. The dose-response curve obtained (Annexe F, Figure F-1) to determine the lethal concentration value (LC_{50}), of this compound showed that at a concentration of 44.49 ± 1.04 mg/ L 50% of *A. saline* tested population were viable.

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. The results are shown in Figure V-4.

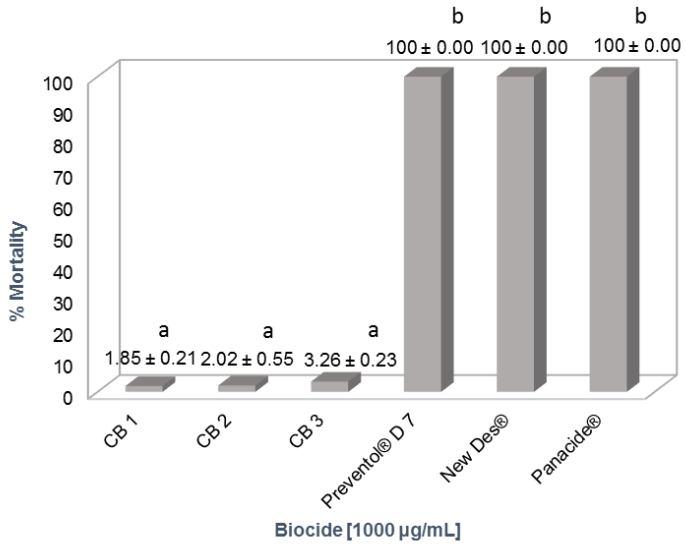


Figure V-4: % of *Artemia salina* mortality for the bioactive compounds produced and for the commercial biocides at a concentration of 1000 µg/mL in each well. Different letters (a-b) following the values indicate significant differences ($p<0.05$). Values of each determination represents means ± SD (n=3).

Whereas no significant differences where observed within the group of the chemical biocides or of the bioactive compounds ($p>0.05$) (Annexe G, Table G-3,4) the differences between both groups are notable.

The differences between the commercial biocides and the antifungal compounds produced are notorious and show no significant differences within the group of the chemical biocides and bioactive compounds. In fact, all the chemical biocides promote the death of the entire *A. salina* population (100 % of mortality) and the compounds produced by *Bacillus* sp. CCLBH 1051 (CB 1) were far less toxic for *A. salina* model (1.85 % ± 0.21).

This assay allows to evaluate the lethal potential effect in previous living organisms and also provides the basis for further analysis with more representative biological models. The Table V-1-1 shows the results of LC_{50} for the different biocides analysed.

Table V-1: LC_{50} parameter 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

The results reveal that Preventol® D 7 requiring a concentration of 5.07 ± 0.35 µg/mL for causing 50% of mortality, exhibits a higher toxicity than NEW DES® (LC_{50} = 34.53 ± 4.70 µg/mL) and Panacide® (LC_{50} = 257.48 ± 8.54 µg/mL).

Among the bioassays most commonly used for preliminary evaluation of extracts of plants and microorganisms, the toxicity on *A. salina* is the most used, because of its

reduce cost and fastness, but also because it does not require aseptic conditions/techniques (Jenkins *et al.*, 1999).

4.1.2. Acute toxicity in *Swiss* mice

The toxicity of the bioactive compounds produced was also evaluated in mammals using *Swiss* mice as biological model and the chemical biocide Preventol® D 7 as positive control.

The results show that the supernatants of *Bacillus* sp. CCLBH 1051, CCLBH 1052 and CCLBH 1053 corresponding to CB1, CB2 and CB3, respectively, did not show lethality according to OECD guideline (OECD, 2001) with LD_{50} values greater than 5000 mg/kg, because no animal died during the experiment.

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 the behaviour (aggression, passivity and fear). The animals showed normal motor, cognitive and sensorial behaviour during the first 24 h of the assay, revealing also no signs of toxicity (Figure V-2).

In the other hand, Preventol® D 7 biocide, used in this assay as positive control, evidenced the death of all animals in the group treated with 1000 and 500 mg/Kg, after 5 to 15 min of administration. However, no animals died from the group treated with 200 mg/kg, where the animals also showed a normal motor, cognitive and sensorial behaviour. Consequently, the LD_{50} of Preventol® D 7 was set between 200 and 500 mg/mL.

Thus, it can be concluded that the new bioactive compounds produced do not induce acute toxicity, motor, cognitive or sensorial alterations at the dose tested, unlike them Preventol® D 7 show lethality when administrated at doses 10 times lower.

This commonly used biocide have a spectrum of activity that covers bacteria, including formaldehyde-resistant species, fungi, yeast and algae, reason for which it is

widely used in conservation/restauration processes. However, its “Safety Data Sheet” described the effects in Human health and Environmental hazards, such as burns and sensitisation by skin contact for humans and long-term adverse effects in the aquatic environment (Lanxess, 2008). For this reason, the handling of Preventol® D 7 required many protection measures that include personal protective equipment, installation of air purification systems and fume extractors in museums and laboratories. Proposing adequate alternative materials and methods of intervention is the key for the eradication of these traditional methods and heath/environment preservation.

The results of this chapter showed the high toxicological intensity level of the commercial biocides in comparison with the lack of toxicity of the natural compounds produced in our laboratory. This confirmed the dangers that the conservators-restorers faced during the restoration processes and the long-term adverse consequences that our environment may face due to the use of commercial traditional biocides. At the same time, the results highlight the benefits of using natural green safe compounds, as those described here.

4.2. New compounds effectiveness in cultural heritage context

4.2.1. Simulation *in vitro* assays using mortar slabs

In order to develop and study the real efficiency and influence of these nontoxic new biocides in the biodeteriogenic fungi development, a combined strategy using manufactured mortars slabs were envisaged. They were inoculated with *Cladosporium* sp. (the most inhibited fungi tested in Chapter III) and treated with produced bioactive compounds by *Bacillus* sp. CCLBH 1053 strain (the most efficient producer bacteria), under *in situ* controlled conditions.

Figure V-5 shows the macroscopic and microscopic features corresponding to the mortar slab assays, in the presence of *Cladosporium* sp.

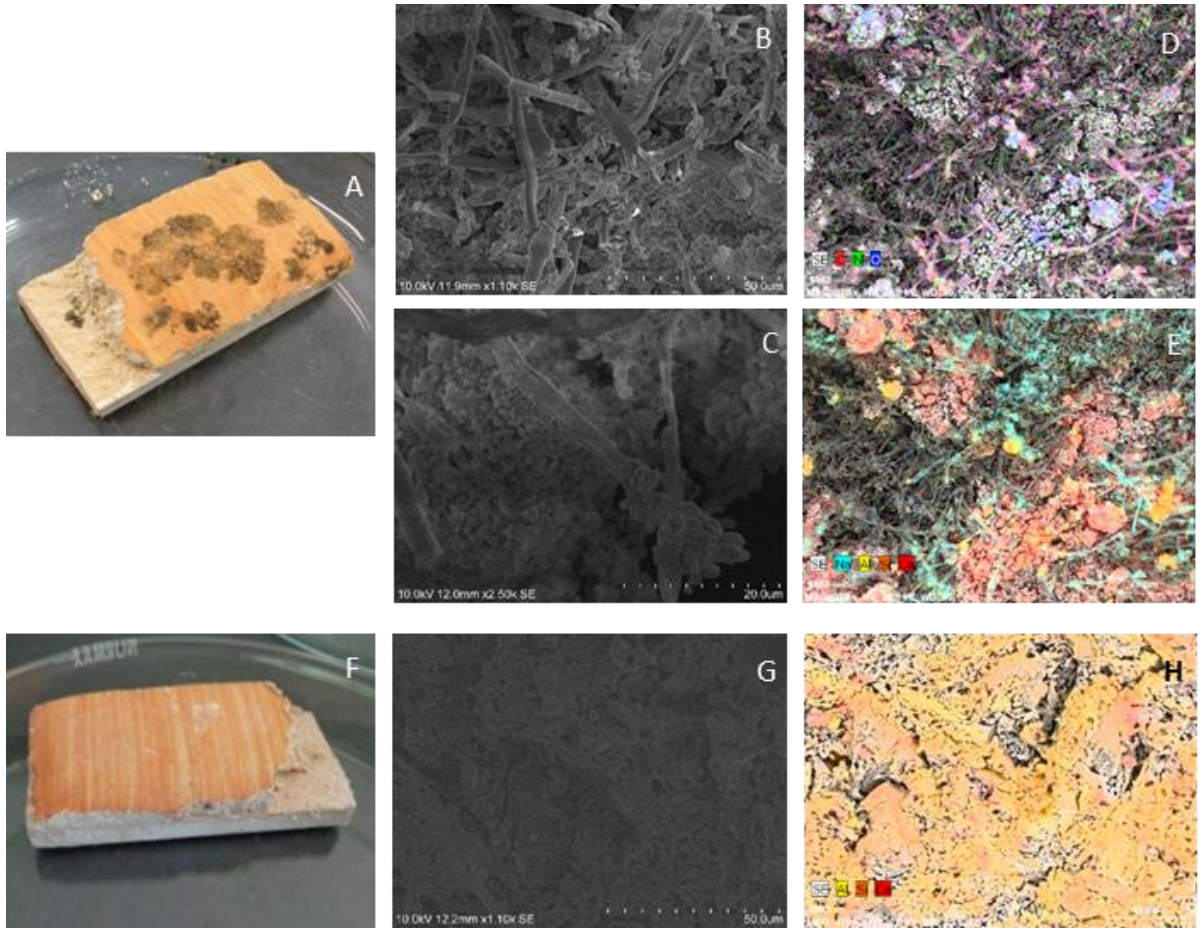


Figure V-5: Mortar slabs, SEM micrographs of the MS1 microfragments and EDS analysis. A-E: control slabs inoculated with *Cladosporium* sp.; F-H: MS with CB 3 (bioactive compounds produced by *Bacillus* sp. CCLBH 1053).

After 2 months of mortar slab treatment, the positive control showed dark stains in the painted surface, indicating a high level of fungal contamination (Figure V-5A). The MS1 assay with CB 3 antifungal treatment presented a very low level of fungal proliferation and no signals of cracking or detachments were detected on the surface of the slabs (Figure V-5F). In order to confirm this 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 (Figure V-5B, C) exhibited a clear presence of microbial cells thriving in the mortar. It was possible to observe *Cladosporium* hyphae and reproductive structures penetrating into the microstructure of the mortars, promoting the proliferation of these microorganisms in depth. Also, the EDS elemental analysis showed the presence of chemical elements such as carbon, nitrogen and oxygen, confirming the presence of organic material in the slab (Figure V-5D, E). Regarding the biocide-treated MS1 slabs, none evidences of fungal growth were observed by the naked eye as well as none spores and hyphae were observed by SEM (Figure V-5F-G). The lack of fungal proliferation was confirmed by the EDS analyses that only showed the presence of mortar nature composition elements, such as silicon (Si), aluminium (Al) and calcium (Ca) (Figure V-5H).

The simulation assays evidenced the great potential of the bacterial bioactive compounds to inhibit fungal proliferation.

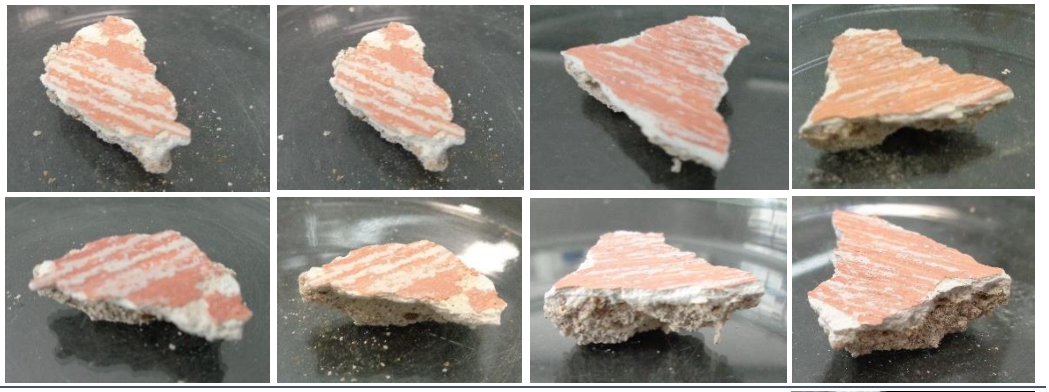
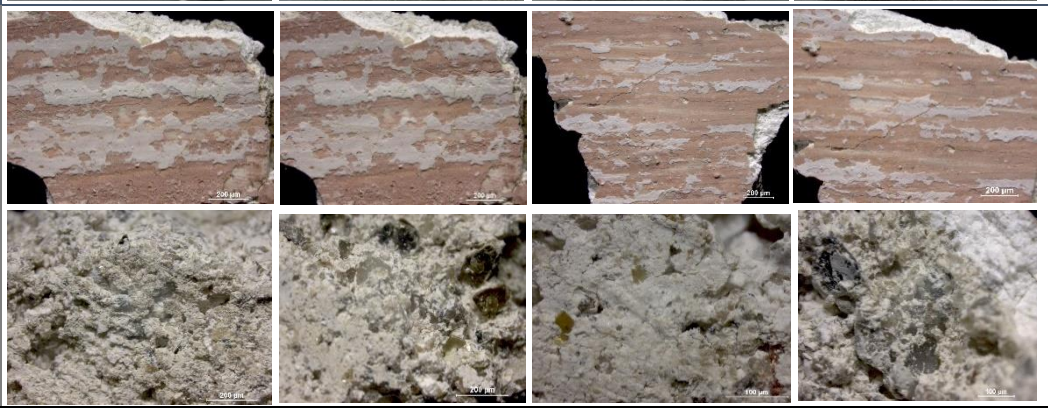
Microbial proliferation on mortars contributes to deterioration of mural paintings. Filamentous fungi development leads the hyphae penetration within the mortar structure, promoting the proliferation of these microorganisms in depth which affects the cohesion of the structure facilitating the appearance of some cracks (Scheerer *et al.*, 2009; Sterflinger, 2010; Rosado *et al.*, 2013a). In this way, the antifungal compounds isolated and tested in this work seem to be a potent alternative to mitigate the problems underneath the fungi proliferation and consequently the physicochemical mechanisms that promote long-term deterioration in artworks with a mortar base construction.

4.2.2. Monitorisation of artificially inoculated fragments of mortars from mural painting

Few studies refer several negative effects 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 (Young *et al.*, 2008), the ineffectiveness of long-term treatment (de los Ríos *et al.* 2012), the absence of selectivity against the target microorganisms (Rakotonirainy *et al.*, 2007; Bastian *et al.*, 2009), discoloration and apparition of other structural damages in the economically important materials (Sasso *et al.*, 2013).

Small and representative fragments of a mural painting from *Santo Aleixo* Church, Montemor-o-Novo, Portugal, were monitored and analysed in the presence of the bioactive compounds produced. This assay allowed to simulate the impact and possible alteration resulting from the new compounds treatment in the microstructure of real mortar and in the painting. Table V-2 shows two pieces of real mortars that were impregnated with extracted compounds (RM1) and cell-free supernatant from *Bacillus* sp. CCLBH 1053 culture (RM2) which proved to be one of the most efficient strain for the bioactive compounds production so far.

Table V-2: Schematic representation of the lab experiments conducted on real mortars samples. RM1- Mortar impregnated with extracted compounds from *Bacillus* sp. CCLBH 1053; RM2- Mortar impregnated with CB 3.

	RM1		***RM2***	
	Before impregnation	After 6 months	Before impregnation	After 6 months
Macroscopical features				
Microscopical features				

After 6 months of incubation under controlled conditions, the two series of mortars do not present any signals of pigment discoloration, cracking or detachments in their surface and structure (Table V-2).

Figure V-6 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.

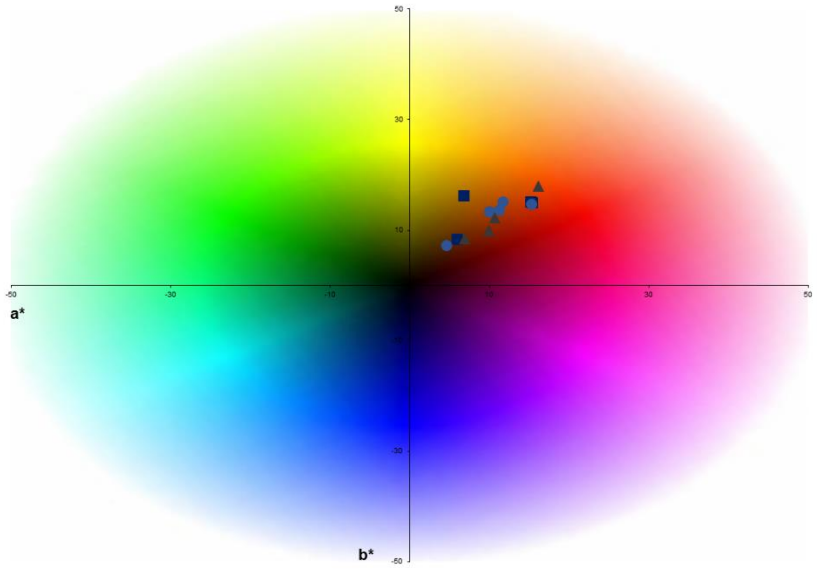


Figure V-6: Bi-dimensional projection of International Commission on Illumination a* b* chromatic coordinates of the 12 measurements that were performed on the real mortars of the Santo Aleixo's mural painting. ▲ Control; ● RM1; ■ RM2.

The results showed a predominance of red for both series and for the control mortar (6<a*<16). None significant differences were found between the control and the treated mortars (p>0.05) (Annexe G, Table G-5, 6). Therefore, these new compounds do not cause any structural and aesthetic damages of both the painting or the mortar, becoming an appropriate treatment with great potential to be applied in cultural and built heritage as remediation or prevention treatment against microbial growth.

Preventive conservation measurements must be performed and a long-term *in situ* monitorisation that encompasses several physical parameters and gathering of possible microorganisms and neoformation products. So, it is important to have in mind that after the conservation/restoration process with any type of biocide it is important to control and prevent possible microbial recolonisation.

Therefore, these new effective compounds, obtained from natural sources and specifically produced against biodeteriogenic agents of architectural, archaeological and artistic heritage, have all the potential to become a ground-breaking solution to be applied in preventive and conservation treatment of artworks.

5. Conclusion

In this last chapter the toxicity level of bioactive compounds produced by *Bacillus* sp. cultures were evaluated in comparison with commercial commonly used biocides.

Brine shrimp (*Artemia salina)* and *Swiss* mice (*Mus musculus*) were used as biological models in order to access a standard test for determination of LC_{50} and acute toxicity for the determination of LD_{50}, respectively.

The natural biocompounds produced in our laboratory with great demonstrated potential to suppress biodeteriogenic fungi growth, exhibited total absence of toxicity against living organisms, namely mammals.

Moreover, in simulation assays using mortars slabs and real samples, the compounds produced by *Bacillus* sp. besides the great potential to suppress biodeteriogenic fungi growth on historical artworks, do not cause any structural and aesthetic damages of both the painting or the mortar.

Therefore, the natural compounds produced by selected *Bacillus* strain prove to have antifungal capacity, structural and aesthetics preserving and low toxicity characteristics. These properties confirmed the green safe promising alternative that these bacterial compounds constituted for future application as additives in new formulations for novel biocides production for biodegradation/biodeterioration treatment of Cultural Heritage.

CHAPTER VI

Concluding Remarks

The main goal of this PhD thesis was to obtain and study new bioactive molecules produced by several bacterial strains obtained from natural sources, in order to establish effective biocides specifically designed to biodeteriogenic agents of artistic heritage.

This research in the area of cultural heritage rehabilitation, by the development of green safe promising alternatives for future application can be used as additives in new formulations of novel biocides for biodegradation/biodeterioration treatment of Cultural Heritage.

The partnership of methodological techniques from Biochemistry, Microbiology and Biotechnology allowed a comprehensive study of the physiological mechanism behind the bioactive compounds production that allows to understand and improve the strategic approaches for production process optimisation.

The *Bacillus* sp. strains, previously isolated in our laboratory, with proven capacity to be producers of green compounds with high commercial potential, were selected through an effective screening methodology that combined antifungal testing with, molecular, microscopic and analytical approaches, without the need of laborious total previous isolation.

The results obtained reveal the high potential of the biological active compounds produced by *Bacillus* sp. to inhibit the proliferation of biodeteriogenic fungi isolated from artistic heritage. Moreover, these compounds proved to be real environmental safe and an innocuous alternative to toxic chemical biocides commonly used by restorers, museum curators and architects during the conservative interventions.

Therefore, the combined methodology used in this work and the strategies outlined proved to be very effective and promising for the detection and characterisation of bioactive compounds produced by *Bacillus* strains, contributing to the development of new mitigation approaches for cultural heritage rehabilitation and safeguard.

The methodology developed for biological active compounds detection, evaluation and characterisation under the framework of this research has proven that:

- The bacteria strains with antifungal potential exhibit the same morphological, biochemical and kinetic characteristics, belonging to the genera *Bacillus;*
- After homology research, the bacteria tested showed higher level of similarity with *Bacilllus subtilis*, *Bacillus amyloliquefaciens* and *Bacillus pumilus* that were confirmed by the phylogenetic relationships comparing the different clusters obtained;
- The antifungal potential is due to the production of bioactive molecules namely lipopeptides (LPP) compounds, mostly from iturin family;
- The gene fragments associated with antibiotic synthesis were efficiently amplified and quantified, confirming the presence and different expression of the iturinic genes into strains, and allowing a quick identification of iturin A-producing strains;
- The variability and expression of the nonribosomal protein synthases responsible for the iturinic compounds biosynthesis allowed the selection of the strains with higher potential for biocontrol;
- Antifungal tests showed high and different levels of inhibition capacity depending on the biodeteriogenic target fungal strain tested and the *Bacillus* strains producer;
- LC-ESI-MS analysis showed, in the case of *Bacillus* sp. cell free supernatant, clusters containing molecules typical of lipopeptides from iturin and fengycin family. These results were also supported by ^{1}H-NMR analysis;
- The combined use of antifungal tests, NMR spectroscopy and MS spectrometry techniques complemented with bioautographic methodology and FTIR-ATR

analyses, suggests that the fengycin and iturin lipopeptides families are the main responsible for the antifungal activity observed;

- The strain of *Bacillus* sp. CCLBH 1053 showed an extraordinary capacity to produce more than one LPP molecule in a suitable amount to be used in coming applications;

- The analysis of microbial cells of *Bacillus* sp. CCLBH 1053 by flow cytometry enhance the knowledge of population dynamics, throughout a bio-process, demonstrating the inherent complex heterogeneity of microbial cultures;

- There was a correlation between the sporulation process and the bioactive compounds production, specifically a new round of lipopeptide production can occur after the beginning of the sporulation; in parallel, peptone supplementation follow a heat shock induced spores germination, promoting a LPP production increment;

- Flow cytometry, and particularly the light scatter parameters gave additional information about the spores/cells distribution and germination process, becoming a useful tool for sporulation monitorisation of *Bacillus* strains;

- Metabolically active cells derived from induced germination of the *Bacillus* spores were responsible for re-production of the iturin and fengycin lipopeptides family after their first metabolic stage;

- The induced spores germination procedure with nutrient supplementation and a heat activation can be apply to the lipopeptides production, constituting a new tool for metabolically activation of spores and improvement of natural metabolites production;

- The bioactive compounds produced offered total absence of toxicity against living organisms, namely mammals;

- Simulation assays showed the real potential of these compounds to reduce and control the growth of fungi involved in biodeterioration process under cultural heritage context.

In order to increase the knowledge about the compounds with antifungal properties produced by *Bacillus* strains and their potential to establish effective biocides specifically designed to biodeteriogenic agents of heritage artefacts, further approaches could be considered.

In this way, we envisage that few complementary studies can be accomplished, including:

- Optimize the scale-up production process using microorganisms with potential to produce antifungal compounds;
- Test of the antagonistic compounds against other biodeteriogenic fungal strains and microbial communities;
- Extend the simulation assays to different materials and complement the real-life efficiency tests;
- Develop new formulations, for different fungal communities, with the antifungal compounds as primary additives.

The development of biocides specific for the biological agents responsible for an artwork deterioration can significantly improve the rehabilitation process and increase the time of protection and conservation of cultural heritage artefacts. This need to be implemented in conservation intervention strategies to promote the safeguard of historical and contemporary artworks.

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ANNEXES

Annexe A . Culture media composition

Table A-1: Composition of the culture media used for microbiological growth.

MEA	ME	NA	NB	CRB	LAPM
30 g/L Malt extract		5 g/L Peptic digest animals		5 g/L Peptone	8 g/L Peptone
5 g/L Mycological peptone		1.5 g/L Beef extract		10g/L Glucose	6.7g/L Maltose
20 g/L Glucose		1.5 g/L Yeast extract		1 g/L K_2HPO_4	0.5 g/L K_2HPO_4
15 g/L Agar	___	5 g/L NaCl		0.5 g/L $MgSO_4$	0.05 g/L $MgSO_4 \cdot 7H_2O$
		15 g/L Agar	___	0.05 g/L Rose Bengal	0.025 g/L $FeSO_4 \cdot 7H_2O$
				0.1 g/L Chloramphenicol	0.022 g/L $MnSO_4 \cdot 7H2O$
				15.5 g/L Agar	0.184 g/L $CaCl_2$

MEA - Malt Extract Agar; ME - Malt Extract; NA – Nutrient Agar; NB – Nutrient Broth; CRB - Cook Rose Bengal; LAPM- Lipopeptide Antibiotic Production Medium

Annexe B . Solutions composition

B1. TBE 10X (pH 8)

- ✓ Tris 890 mM
- ✓ Boric acid 890 mM
- ✓ EDTA 0.5 M

B2. TBE 1x (pH 8)

- ✓ TBE 10x diluted 1/10 in sterile water

Annexe C . *Bacillus* 16S ribosomal DNA amplification and agarose gel electrophoresis

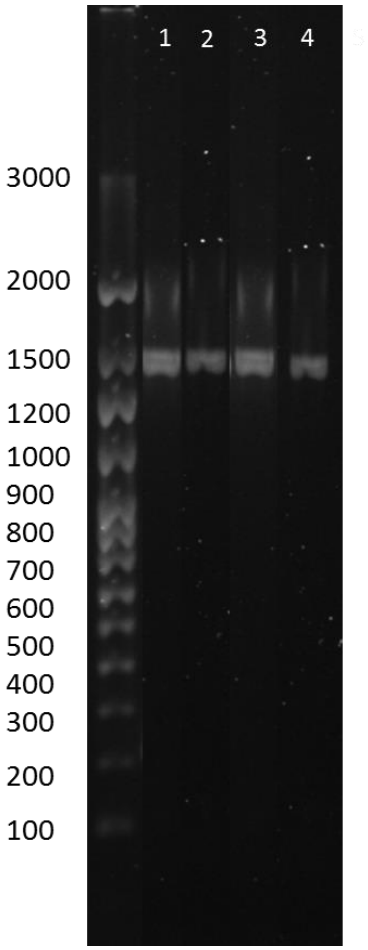


Figure C-1: Agarose gel electrophoresis of 16S ribosomal DNA isolated from different *Bacillus* sp. strains. Legend: Lane 1- 100 bp Ladder (Nzytech Ladder VII); Lane 2- PCR product of 16S rDNA amplification of *Bacillus* sp. CCLBH 1051; Lane 3- PCR product of 16S rDNA amplification of *Bacillus* sp. CCLBH 1052; Lane 4- PCR product of 16S rDNA amplification of *Bacillus* sp. CCLBH 1053; Lane 5- PCR product of 16S rDNA amplification of *Bacillus* sp. CCLBH 1054.

Annexe D . Determination of DNA extracted from the bacteria cells

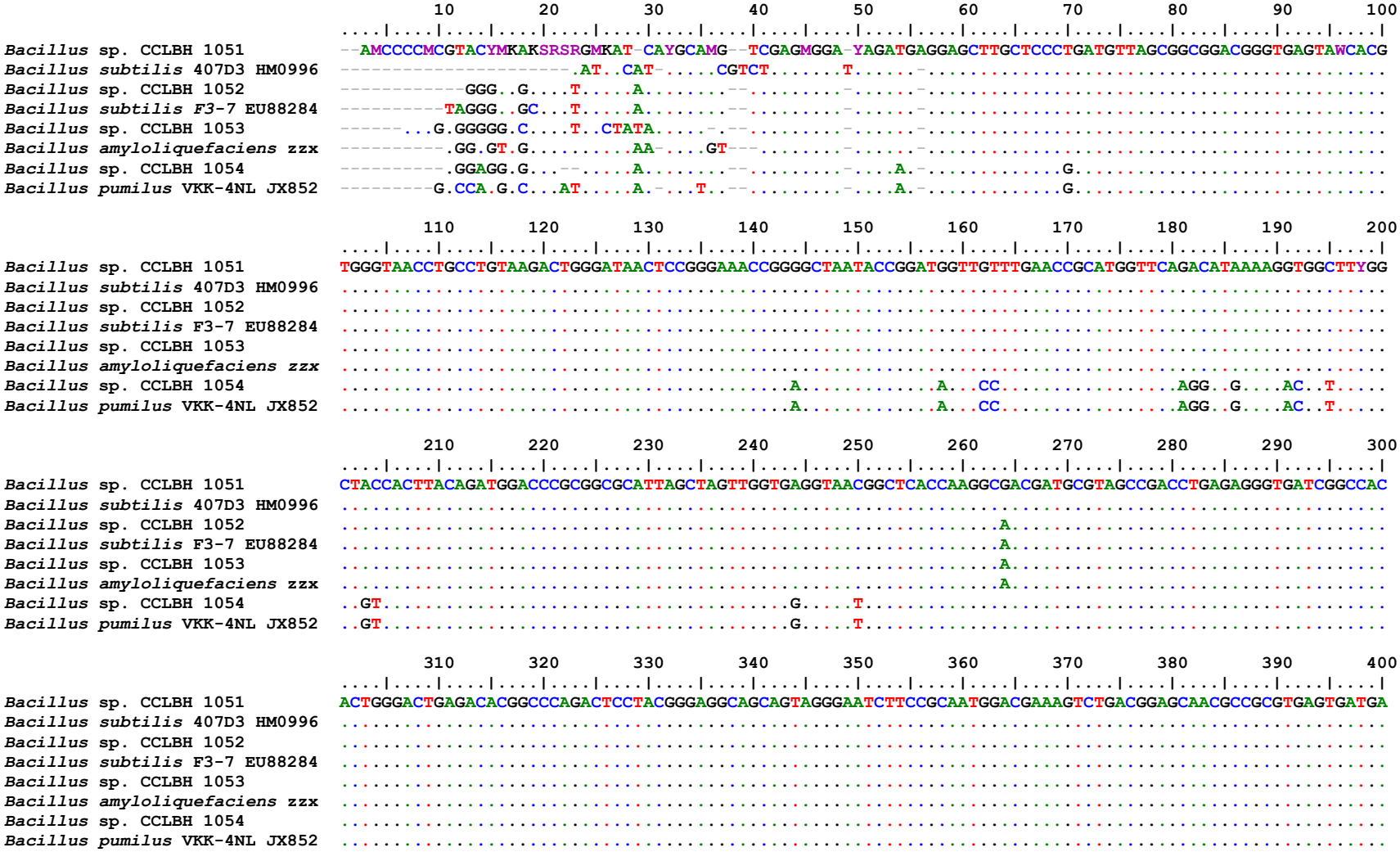
The concentration and purity of the DNA extracted from the *Bacillus* sp. cells were determined using spectrophotometric analysis. The Table D-1 shows the different concentration of the bacterial DNA.

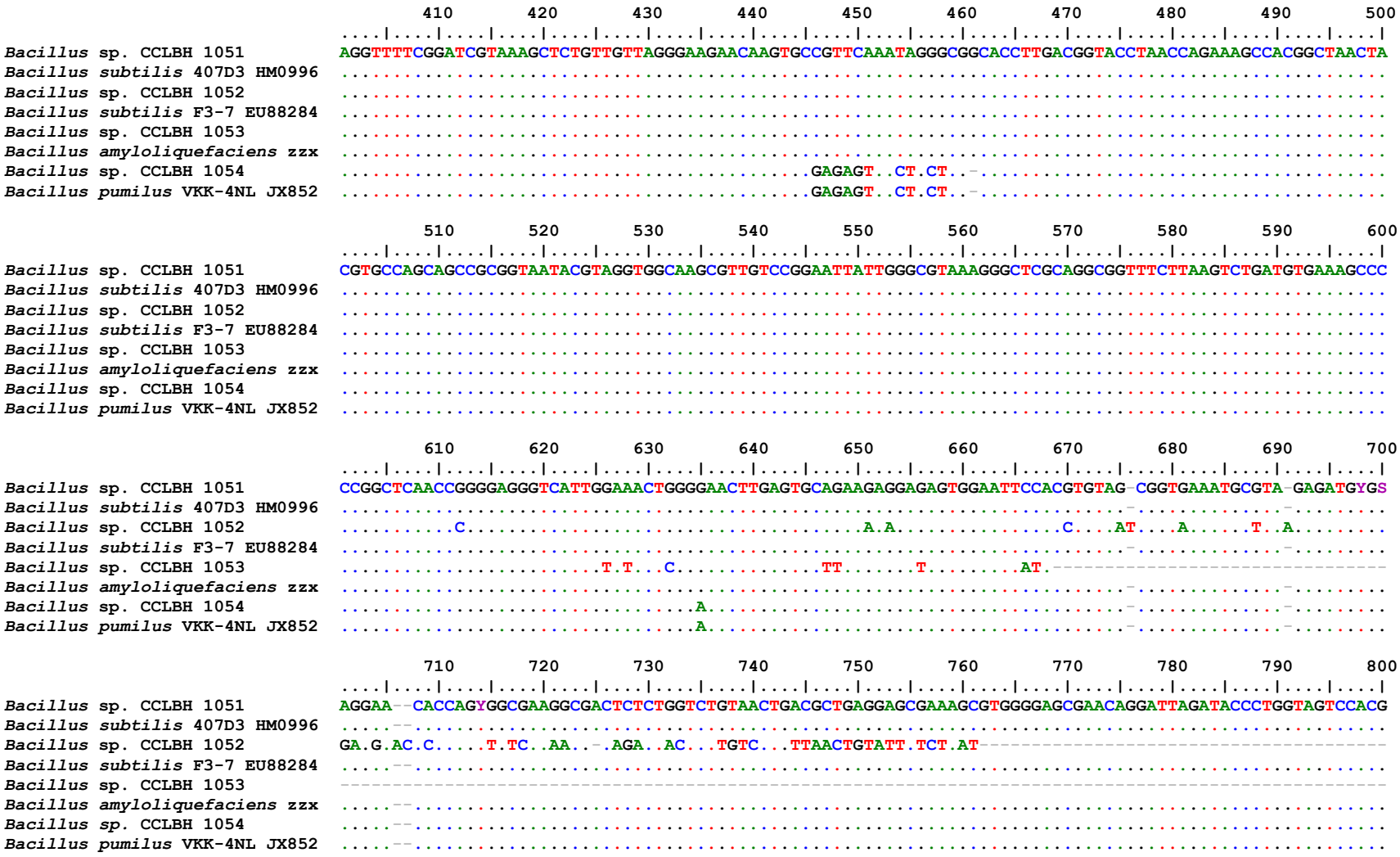
Table D-1: DNA concentration extracted from of the bacteria strains in analysis.

***Bacillus* sp.**	**DNA concentration (µg/mL)**
CCLBH 1051	11.83 ± 1.91
CCLBH 1052	22.55 ± 1.03
CCLBH 1053	16.68 ± 0.90
CCLBH 1054	22.10 ± 1.89

By spectrophotometry, in addition to determining the DNA concentration, the A260/280 can be used to assess the DNA purity concerning the protein contamination. Pure DNA preparations have an A260/280 of 1.8. Similarly, absorbance at 230 nm decreases the values of A260/230 and indicates contamination by polyphenols compounds. For the DNA extracted, the ratio A260/280 present values higher than 1.8. Low values of A260/230 ration were also obtained indicated the low organic compounds contamination.

Annexe E . Multiple alignment of the rDNA 16s sequence of the *Bacillus* sp. strains and the most similar *Bacillus* sequence found for each one in the Gene Bank sequence database.

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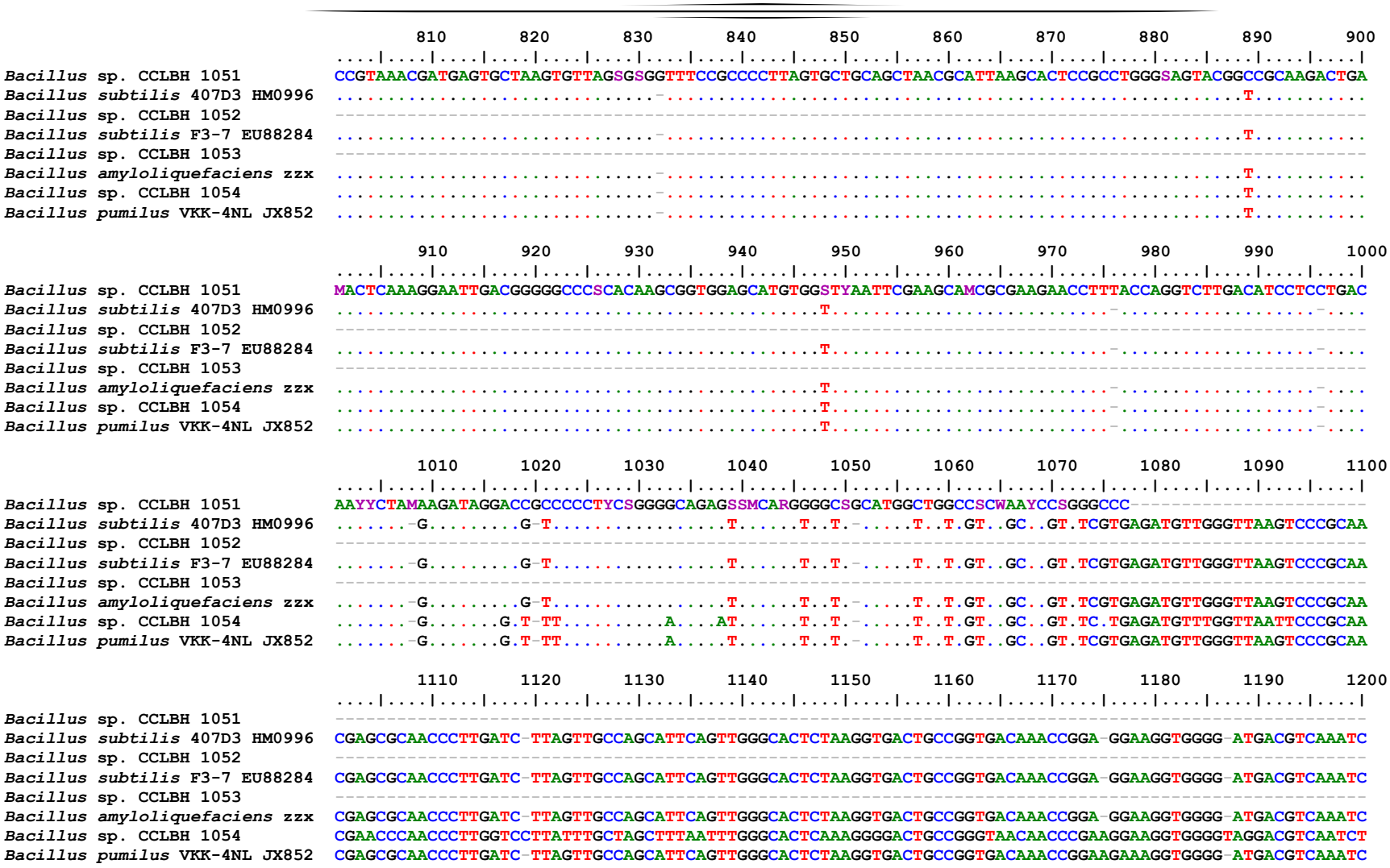
Bacillus sp. CCLBH 1051 AGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTA
Bacillus subtilis 407D3 HM0996
Bacillus sp. CCLBH 1052
Bacillus subtilis F3-7 EU88284
Bacillus sp. CCLBH 1053
Bacillus amyloliquefaciens zzx
Bacillus sp. CCLBH 1054
Bacillus pumilus VKK-4NL JX852
510 520 530 540 550 560 570 580 590 600
Bacillus sp. CCLBH 1051 CGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCC
Bacillus subtilis 407D3 HM0996
Bacillus sp. CCLBH 1052
Bacillus subtilis F3-7 EU88284
Bacillus sp. CCLBH 1053
Bacillus amyloliquefaciens zzx
Bacillus sp. CCLBH 1054
Bacillus pumilus VKK-4NL JX852
610 620 630 640 650 660 670 680 690 700
Bacillus sp. CCLBH 1051 CCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAG-CGGTGAAATGCGTA-GAGATGYGS
Bacillus subtilis 407D3 HM0996
Bacillus sp. CCLBH 1052
Bacillus subtilis F3-7 EU88284
Bacillus sp. CCLBH 1053
Bacillus amyloliquefaciens zzx
Bacillus sp. CCLBH 1054
Bacillus pumilus VKK-4NL JX852
710 720 730 740 750 760 770 780 790 800
Bacillus sp. CCLBH 1051 AGGAA--CACCAGYGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACG
Bacillus subtilis 407D3 HM0996
Bacillus sp. CCLBH 1052
Bacillus subtilis F3-7 EU88284
Bacillus sp. CCLBH 1053
Bacillus amyloliquefaciens zzx
Bacillus sp. CCLBH 1054
Bacillus pumilus VKK-4NL JX852


810 820 830 840 850 860 870 880 890 900
Bacillus sp. CCLBH 1051
Bacillus subtilis 407D3 HM0996
Bacillus sp. CCLBH 1052
Bacillus subtilis F3-7 EU88284
Bacillus sp. CCLBH 1053
Bacillus amyloliquefaciens zzx
Bacillus sp. CCLBH 1054
Bacillus pumilus VKK-4NL JX852
910 920 930 940 950 960 970 980 990 1000
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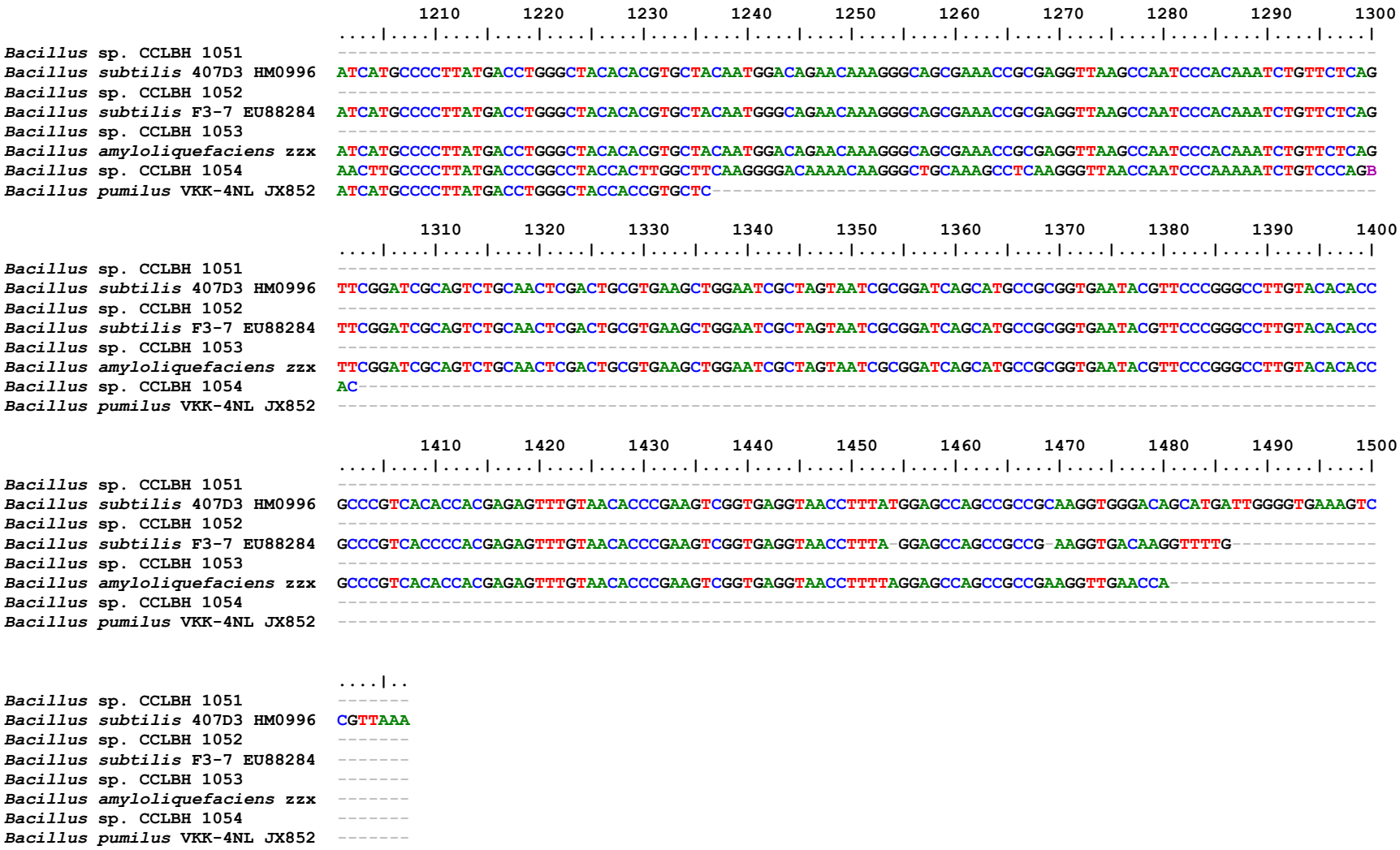
1110 1120 1130 1140 1150 1160 1170 1180 1190 1200


1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
Bacillus sp. CCLBH 1051
Bacillus subtilis 407D3 HM0996 ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAG
Bacillus sp. CCLBH 1052
Bacillus subtilis F3-7 EU88284 ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAG
Bacillus sp. CCLBH 1053
Bacillus amyloliquefaciens zzx ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAG
Bacillus sp. CCLBH 1054 AACTTGCCCCTTATGACCCGGCCTACCACTTGGCTTCAAGGGGACAAAACAAGGGCTGCAAAGCCTCAAGGGTTAACCAATCCCAAAAATCTGTCCCAGB
Bacillus pumilus VKK-4NL JX852 ATCATGCCCCTTATGACCTGGGCTACCACCGTGCTC
1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
Bacillus sp. CCLBH 1051
Bacillus subtilis 407D3 HM0996 TTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC
Bacillus sp. CCLBH 1052
Bacillus subtilis F3-7 EU88284 TTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC
Bacillus sp. CCLBH 1053
Bacillus amyloliquefaciens zzx TTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC
Bacillus sp. CCLBH 1054 AC
Bacillus pumilus VKK-4NL JX852
1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
Bacillus sp. CCLBH 1051
Bacillus subtilis 407D3 HM0996 GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTATGGAGCCAGCCGCCGCAAGGTGGGACAGCATGATTGGGGTGAAAGTC
Bacillus sp. CCLBH 1052
Bacillus subtilis F3-7 EU88284 GCCCGTCACCCCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTA-GGAGCCAGCCGCCG-AAGGTGACAAGGTTTTG
Bacillus sp. CCLBH 1053
Bacillus amyloliquefaciens zzx GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAGGTTGAACCA
Bacillus sp. CCLBH 1054
Bacillus pumilus VKK-4NL JX852
Bacillus sp. CCLBH 1051
Bacillus subtilis 407D3 HM0996 CGTTAAA
Bacillus sp. CCLBH 1052
Bacillus subtilis F3-7 EU88284
Bacillus sp. CCLBH 1053
Bacillus amyloliquefaciens zzx
Bacillus sp. CCLBH 1054
Bacillus pumilus VKK-4NL JX852

Annexe F. Toxicity assay

F1. *Artemia salina* medium composition

Table F-1: Composition of the saline medium used to *Artemia salina* toxicity assay.

Saline medium
3.0 g/L $NaHCO_3$
30.0 g/L of NaCl
0.3 g/L $CaCl_2$
0.5 g/L $MgSO_4.7H_20$
1.5 g/L $MgCl_2$
0.8 g/L KCl
0.1 g/L H_3BO_3

F2. Potassium dichromate dose- response curve

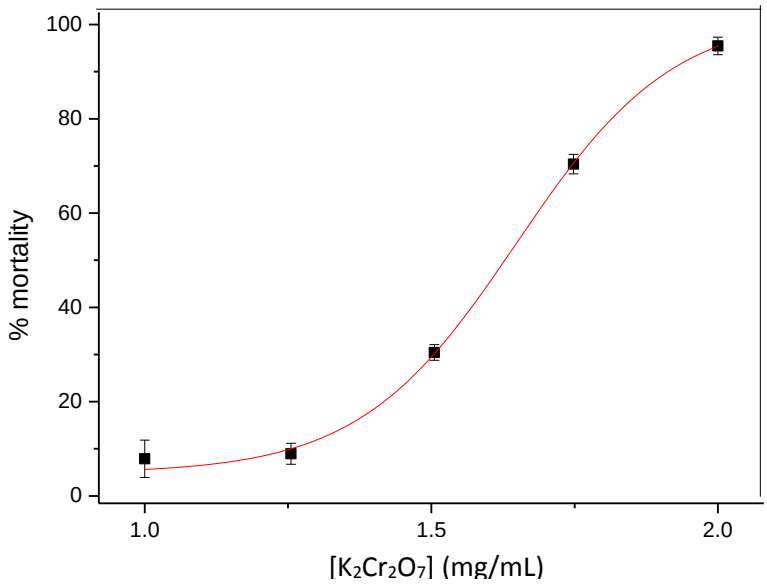


Figure F-1: Potassium dichromate dose-response curve for *Artemia salina* assay.

Annexe G . Statistical analysis

Table G-1: Analysis of variance (ANOVA) of interaction assay against biodeteriogenic fungi.

		Sum of Squares	df	Mean Square	F	Sig.
Penicillium sp.	Between Groups	32.303	2	16.151	4.392	.067
	Within Groups	22.064	6	3.677		
	Total	54.367	8			
Alternaria sp.	Between Groups	330.212	2	165.106	13.603	.006
	Within Groups	72.826	6	12.138		
	Total	403.038	8			
Mucor sp.	Between Groups	191.011	2	95.506	.889	.459
	Within Groups	644.589	6	107.431		
	Total	835.600	8			
Fusarium oxysporum	Between Groups	301.216	2	150.608	61.968	.000
	Within Groups	14.583	6	2.430		
	Total	315.798	8			
Penicillium sp.2	Between Groups	593.455	2	296.727	13.592	.006
	Within Groups	130.983	6	21.830		
	Total	724.437	8			
Cladosporium sp.	Between Groups	3200.000	2	1600.000	3.000	.125
	Within Groups	3200.000	6	533.333		
	Total	6400.000	8			
Aspergillus niger	Between Groups	3396.604	2	1698.302	1.793	.245
	Within Groups	5684.396	6	947.399		
	Total	9081.000	8			

Table G-2: Average multiple comparison of interaction liquid assay against biodeteriogenic fungi, by Tukey HSD test.

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Penicillium sp.1	CCLBH 1051	CCLBH 1052	-2.58667	1.56575	.297	-7.3908	2.2175
		CCLBH 1053	-4.63000	1.56575	.057	-9.4341	.1741
	CCLBH 1052	CCLBH 1051	2.58667	1.56575	.297	-2.2175	7.3908
		CCLBH 1053	-2.04333	1.56575	.443	-6.8475	2.7608
	CCLBH 1053	CCLBH 1051	4.63000	1.56575	.057	-.1741	9.4341
		CCLBH 1052	2.04333	1.56575	.443	-2.7608	6.8475
Alternaria sp.	CCLBH 1051	CCLBH 1052	14.83667*	2.84460	.005	6.1086	23.5647
		CCLBH 1053	7.52333	2.84460	.085	-1.2047	16.2514
	CCLBH 1052	CCLBH 1051	-14.83667*	2.84460	.005	-23.5647	-6.1086
		CCLBH 1053	-7.31333	2.84460	.093	-16.0414	1.4147
	CCLBH 1053	CCLBH 1051	-7.52333	2.84460	.085	-16.2514	1.2047
		CCLBH 1052	7.31333	2.84460	.093	-1.4147	16.0414
Mucor sp.	CCLBH 1051	CCLBH 1052	4.62264	8.46292	.852	-21.3439	30.5892
		CCLBH 1053	11.22642	8.46292	.433	-14.7401	37.1930
	CCLBH 1052	CCLBH 1051	-4.62264	8.46292	.852	-30.5892	21.3439
		CCLBH 1053	6.60377	8.46292	.728	-19.3628	32.5703
	CCLBH 1053	CCLBH 1051	-11.22642	8.46292	.433	-37.1930	14.7401
		CCLBH 1052	-6.60377	8.46292	.728	-32.5703	19.3628
Fusarium oxysporum	CCLBH 1051	CCLBH 1052	5.35667*	1.27291	.013	1.4510	9.2623
		CCLBH 1053	14.04000*	1.27291	.000	10.1344	17.9456
	CCLBH 1052	CCLBH 1051	-5.35667*	1.27291	.013	-9.2623	-1.4510
		CCLBH 1053	8.68333*	1.27291	.001	4.7777	12.5890
	CCLBH 1053	CCLBH 1051	-14.04000*	1.27291	.000	-17.9456	-10.1344
		CCLBH 1052	-8.68333*	1.27291	.001	-12.5890	-4.7777
Penicillium sp.2	CCLBH 1051	CCLBH 1052	19.89000*	3.81492	.005	8.1848	31.5952
		CCLBH 1053	10.08000	3.81492	.085	-1.6252	21.7852
	CCLBH 1052	CCLBH 1051	-19.89000*	3.81492	.005	-31.5952	-8.1848
		CCLBH 1053	-9.81000	3.81492	.093	-21.5152	1.8952
	CCLBH 1053	CCLBH 1051	-10.08000	3.81492	.085	-21.7852	1.6252
		CCLBH 1052	9.81000	3.81492	.093	-1.8952	21.5152
Cladosporium sp.	CCLBH 1051	CCLBH 1052	-40.00000	18.85618	.165	-97.8559	17.8559
		CCLBH 1053	-40.00000	18.85618	.165	-97.8559	17.8559
	CCLBH 1052	CCLBH 1051	40.00000	18.85618	.165	-17.8559	97.8559
		CCLBH 1053	0.00000	18.85618	1.000	-57.8559	57.8559
	CCLBH 1053	CCLBH 1051	40.00000	18.85618	.165	-17.8559	97.8559
		CCLBH 1052	0.00000	18.85618	1.000	-57.8559	57.8559

Aspergillus niger	CCLBH 1051	CCLBH 1052	-44.95333	25.13165	.251	-122.0641	32.1575
		CCLBH 1053	-8.96000	25.13165	.933	-86.0708	68.1508
	CCLBH 1052	CCLBH 1051	44.95333	25.13165	.251	-32.1575	122.0641
		CCLBH 1053	35.99333	25.13165	.384	-41.1175	113.1041
	CCLBH 1053	CCLBH 1051	8.96000	25.13165	.933	-68.1508	86.0708
		CCLBH 1052	-35.99333	25.13165	.384	-113.1041	41.1175

*. The mean difference is significant at the 0.05 level.

Table G-3: Analysis of variance (ANOVA) of the *Artemia salina* toxicity assay for bioactive compounds produced and commercial biocides.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	42888.343	5	8577.669	2164.917	.000
Within Groups	47.545	12	3.962		
Total	42935.889	17			

Table G-4: Average multiple comparison of the *Artemia salina* toxicity assay for bioactive compounds produced and commercial biocides.

Dependent Variable		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CB1	CB2	-.17021	1.62524	1.000	-5.6293	5.2889
	CB3	-1.40980	1.62524	.947	-6.8689	4.0493
	Preventol D 7	-98.14815*	1.62524	.000	-103.6072	-92.6891
	New Des	-98.14815*	1.62524	.000	-103.6072	-92.6891
	Panacide	-98.14815*	1.62524	.000	-103.6072	-92.6891
CB2	CB1	.17021	1.62524	1.000	-5.2889	5.6293
	CB3	-1.23959	1.62524	.969	-6.6987	4.2195
	Preventol D 7	-97.97794*	1.62524	.000	-103.4370	-92.5189
	New Des	-97.97794*	1.62524	.000	-103.4370	-92.5189
	Panacide	-97.97794*	1.62524	.000	-103.4370	-92.5189
CB3	CB1	1.40980	1.62524	.947	-4.0493	6.8689
	CB2	1.23959	1.62524	.969	-4.2195	6.6987
	Preventol D 7	-96.73835*	1.62524	.000	-102.1974	-91.2793
	New Des	-96.73835*	1.62524	.000	-102.1974	-91.2793
	Panacide	-96.73835*	1.62524	.000	-102.1974	-91.2793
Preventol D 7	CB1	98.14815*	1.62524	.000	92.6891	103.6072
	CB2	97.97794*	1.62524	.000	92.5189	103.4370
	CB3	96.73835*	1.62524	.000	91.2793	102.1974
	New Des	0.00000	1.62524	1.000	-5.4591	5.4591
	Panacide	0.00000	1.62524	1.000	-5.4591	5.4591
New Des	CB1	98.14815*	1.62524	.000	92.6891	103.6072
	CB2	97.97794*	1.62524	.000	92.5189	103.4370
	CB3	96.73835*	1.62524	.000	91.2793	102.1974
	Preventol D 7	0.00000	1.62524	1.000	-5.4591	5.4591
	Panacide	0.00000	1.62524	1.000	-5.4591	5.4591
Panacide	CB1	98.14815*	1.62524	.000	92.6891	103.6072
	CB2	97.97794*	1.62524	.000	92.5189	103.4370
	CB3	96.73835*	1.62524	.000	91.2793	102.1974
	Preventol D 7	0.00000	1.62524	1.000	-5.4591	5.4591
	New Des	0.00000	1.62524	1.000	-5.4591	5.4591

*. The mean difference is significant at the 0.05 level.

Table G-5: Analysis of variance (ANOVA) of the bi-dimensional projection of CIElab chromatic coordinates of the real mortars from mural painting.

		Sum of Squares	df	Mean Square	F	Sig.
L	Between Groups	38.933	2	19.467	.455	.645
	Within Groups	512.940	12	42.745		
	Total	551.874	14			
a	Between Groups	20.615	2	10.308	.430	.660
	Within Groups	287.340	12	23.945		
	Total	307.955	14			
b	Between Groups	18.653	2	9.327	.518	.608
	Within Groups	215.895	12	17.991		
	Total	234.548	14			

Table G-6: Average multiple comparison of the bi-dimensional projection of CIEla*b* chromatic coordinates, by Tukey HSD test.

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
L	Controlo	RM1	3.822000	4.134975	.636	-7.20955	14.85355
		RM2	1.060000	4.134975	.965	-9.97155	12.09155
	RM1	Controlo	-3.822000	4.134975	.636	-14.85355	7.20955
		RM2	-2.762000	4.134975	.786	-13.79355	8.26955
	RM2	Controlo	-1.060000	4.134975	.965	-12.09155	9.97155
		RM1	2.762000	4.134975	.786	-8.26955	13.79355
a	Controlo	RM1	1.052000	3.094832	.939	-7.20459	9.30859
		RM2	2.840000	3.094832	.640	-5.41659	11.09659
	RM1	Controlo	-1.052000	3.094832	.939	-9.30859	7.20459
		RM2	1.788000	3.094832	.834	-6.46859	10.04459
	RM2	Controlo	-2.840000	3.094832	.640	-11.09659	5.41659
		RM1	-1.788000	3.094832	.834	-10.04459	6.46859
b	Controlo	RM1	-.170000	2.682627	.998	-7.32688	6.98688
		RM2	2.276000	2.682627	.681	-4.88088	9.43288
	RM1	Controlo	.170000	2.682627	.998	-6.98688	7.32688
		RM2	2.446000	2.682627	.643	-4.71088	9.60288
	RM2	Controlo	-2.276000	2.682627	.681	-9.43288	4.88088
		RM1	-2.446000	2.682627	.643	-9.60288	4.71088

*. The mean difference is significant at the 0.05 level.