INTERNATIONAL JOURNAL OF CONSERVATION SCIENCE

ISSN: 2067-533X Volume 4, Special Issue, 2013: 603-612 www.ijcs.uaic.ro

ENZYMATIC MONITORIZATION OF MURAL PAINTINGS BIODEGRADATION AND BIODETERIORATION

Tânia ROSADO[1,2,3], Maria Rosário MARTINS[1,3,4], Margarida PIRES[1], José MIRÃO[3,5], António CANDEIAS[1,2,3], Ana Teresa CALDEIRA[1,2,3*]

[1] Chemistry Department, Évora University, Évora, Portugal
[2] Évora Chemistry Centre Chemistry Department, Evora University, Évora, Portugal
[3] HERCULES Laboratory, Évora University, Évora, Portugal
[4]ICAAM - Institute of Mediterranean Agricultural and Environmental Sciences, Évora University, Évora, Portugal
[5]Évora Geophysics Centre and Geosciences Department, Évora University, Évora, Portugal

Abstract

Biodegradation and biodeterioration of mural paintings by microorganisms is an important research field that needs novel approaches to fully understand the mechanisms and effects. In this work, the presence of microorganisms and their biological activity were investigated by extra and intracellular enzymatic monitorization. The enzymes arylsulphatase, β-glucosidase, dehydrogenase and phosphatase were used as biomarkers of the microbial metabolic activity. The viability cellular assays revealed a relationship with the degradation levels of the paintings. In this way, the metabolic activity of the microbial population can be correlated with the contamination levels detected and with biodegradation and biodeterioration status of the paintings. Therefore, enzymatic approaches constitute good biomarkers to be applied in this research field and are useful to detect biodeteriogenic and biodegradation agents.

Keywords: *Mural paintings; Biodegradation; Biodeterioration; Enzymatic monitorization; Viability assays*

Introduction

Mural painting is not only a form of art but also a way to learn more about our ancestral traditions. Biodegradation and biodeterioration is a serious risk to Cultural Heritage, which needs the application of effective and fast methods in order to identify the microorganisms involved in this process and to assess their biodegradation and biodeterioration ability [1]. The term biodeterioration is defined as unwanted alteration in a material caused by the activity of biological agents. Biodeteriogenic organisms have the ability to use a substrate to sustain their growth and reproduction, producing alterations [2]. Several microorganisms can grow on various materials, causing their biodegradation and biodeterioration. In fact it is a complex process that illustrates the interaction of living microorganisms with its substratum and environment [3]. Some microorganisms have the capacity to degrade mural paintings and its biodeteriogenic ability in synergy with other physical and chemical agents may increase the damages [4, 5].

The microbial flora present in artworks, like mural paintings, may result from the successive colonisations by different microorganisms. Their biological attack occurs at favourable temperature and relative humidity conditions for the development of

* Corresponding author: atc@uevora.pt

microorganisms and spores present on the substratum. Each colonizer agent has different ways to compromise the structure in function of the substrates [6, 7].

Microorganisms that grow on mural paintings may origin structural damages involving different processes, such as cracking, exfoliation of paint layers, formation of paint blisters and detachment of the paint layer from the support, or, aesthetic damages which involves the pigment discoloration and stains. It is believed that aesthetic damages occur earlier than structural damages and can precede serious corruption of the materials, being these damages strongly linked [8, 9].

Usually, the study of microflora involved in biodegradation and biodeterioration processes, was based on DNA-dependent methods or in isolation procedures that were mainly useful to provide information about the presence of microbial communities. However, the physiological/biological potential of these microorganisms has not been explored [10]. Therefore, enzymatic studies must be taken into account to understand the role of their metabolic activity on the biodegradation and biodeterioration process.

The ability of enzymes to recognize specific molecules as substrates has led to the proposal of enzyme-based analytical approaches. In this work, different enzymatic systems: arylsulphatase, dehydrogenase, β-glucosidase and phosphatase were chosen to assess the physiological features of the predominant mural painting colonisers and to evaluate their biodegradative and biodeteriorative potential. The enzymes β-glucosidase, phosphatase and arylsulphatase, hydrolyse and catalyse specific reactions involved in the biogeochemical transformations of carbon (C), phosphorus (P) and sulphur (S). These enzymes regenerate inorganic nutrients from organic materials and have been reported as the rate-limiting step in the nutrient cycling process. On the other hand, organic phosphorus (P) must be mineralized into inorganic orthophosphate (PO_4^{3-}) ions to be assimilated by many organisms. Only enzymes produced by plants and/or microorganisms are able to hydrolyse organic P into phosphates. Dehydrogenase enzyme, detect viable organisms and can be considered an accurate measure of the microbial oxidative activity [11, 12]**.**

The main goal of this work was to access the presence of microorganisms and evaluate their effect in the mural paintings deterioration, using extra and intracellular enzymes, as biomarkers of the degradation assisted or not by deterioration process.

Materials and Methods

Selected Microrganisms

Microbiological specimens used (*Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp. and *Rhodotorula* sp.), were isolated from mural paintings belonging to the microorganisms laboratory collection (HERCULES-Biotech Laboratory, Évora University).

Samples

Mortar fragments from a contaminated historical mural painting were collected with sterile scalpels and microtubes, in two areas of the painting with different degradation and deterioration levels.

Analysis of mortar fragments

Mortar samples were analysed by scanning electron microscopy (SEM) in order to assess the degree of degradation and deterioration of the support and the type of colonizing microorganisms. The samples were gold sputtered (Balzers Union SCD030) and then observed under a scanning electron microscope (Hitachi 3700N) operated at high vacuum with an accelerating voltage 10–20 kV in secondary electron mode.

In vitro simulation of mortars colonisations

To evaluate the role of the microorganisms in the mural painting degradation and deterioration, a combinatory strategy was used:

a) Development of liquid cultures with high cells density of pure cultures: *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp. and *Rhodotorula* sp., and a mix culture with these microorganisms
b) Simulation of the influence of these microorganisms on real sterilised mortars microsamples, with isolate and combined cultures.

Fresh cultures of fungi were prepared in solid medium – MEA (Malt Extract Agar). Fungal suspensions were prepared washing each slant with 2mL of NaCl 0.85% solution. The suspensions from pure cultures and a mixed culture obtained by the combination of the four isolates were inoculated in 100mL of Malt Extract liquid medium and incubated at 28ºC in an orbital shaker at 150rpm (Heidolph unimax 1010), during 15 days.

For the simulation assays, mortar samples were sterilized, 1000 mg were distributed in Petri dishes and inoculated with 0.2mL of the suspension prepared as the same form mentioned above (pure and mixed cultures) and incubated at 28ºC during 15 days.

Enzymatic assays

The enzymes arylsulphatase, β-glucosidase, phosphatase and dehydrogenase were monitored in the assays of liquid cultures, in the simulations assays and in real mortar samples.

Arylsulfatase activity was assayed according to the method of Tabatabai and Bremmer [13]. The liquid (0.3mL) and solid (0.1g) samples were incubated 2h at 20ºC with 0.5M acetate buffer pH 5.8 and 0.2mL of 2mM *p*-nitrophenyl sulphate (PNS). The reaction was stopped by adding 0.1mL of 0.5M NaOH, and immediately centrifuged for 15min at 10,000rpm. The amount of *p*-nitrophenol (*p*-NP) released from PNS was measured spectrophotometrically in the supernatant at 405 nm.

β-Glucosidase activities were also evaluated according to the method of Tabatabai and Bremmer [13]. The liquid (0.1mL) and solid (0.1g) samples were incubated with modified universal buffer (MUB) pH 6.0 and 0.2mL of 2mM *p*-nitrophenyl-β-D-glucoside, during 1h at 37ºC. The reaction was stopped by adding 0.1mL of 0.5mM NaOH, and centrifuged for 15 min at 10,000rpm. The amount of *p*-nitrophenol released was measured in the supernatant at 405 nm.

The enzymatic activity of the phosphatase was evaluated according to the method of Tabatabai and Bremmer [14]. The liquid (0.1mL) and solid (0.1g) samples were incubated at 37ºC for 1h modified universal buffer (MUB) pH 5.0 and 0.2mL of 115mM *p*-nitrophenyl phosphate (*p*-PNP). The reaction was stopped by adding 0.1mL of 0.5M NaOH, and immediately centrifuged for 15 min at 10,000rpm. The amount of *p*-nitrophenol released from PNP was measured in the supernatant at 405nm.

Dehydrogenase enzymatic activity was determined according to Camiña [12, 15]. The liquid (0.1mL) and solid (0.1g) samples were incubated for 1h at 40ºC, in the dark, with 1 M of Tris-HCl buffer pH 7.5 and 0.2mL of 0.5% 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT). The reaction was stopped by adding 0.1mL of ethanol/DMF (1:1), and immediately centrifuged for 15min at 10,000rpm. The amount of iodonitrotetrazolium formazan (INTF) released was measured spectrophotometrically in the supernatant at 490 nm.

These assays were performed in triplicate. A unit (U) of enzyme activity was defined as µmole of substrate hydrolysed or oxidized min^{-1}, and per mg of protein for liquid assays or per mg of mortar for solid assays.

Statistical analyses

The results were reported as means ± standard deviation (SD). Data were evaluated statistically using the SPSS® 20.0 software, by descriptive parameters and by ANOVA *One-way*

in order to determine statistically significant differences at the 95% confidence level ($p < 0.05$). Multiple comparisons of average were evaluated by Tukey test.

Cell viability analyses

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described by Mosmann [16].

A 0.1g of mortar samples from mural paintings sites with different degradation levels were incubated with 0.5mL of MTT stock solution (5mg/mL) during 3h, at room temperature, in the dark. After this period, 1mL of ethanol/DMF (1:1) was added to dissolve the formazan crystals formed. The final suspension was centrifuged at 10,000rpm for 15min and the supernatant was spectrophotometrically analysed at 570nm. Each assay was performed in triplicate.

Results and Discussion

Previous studies have revealed that mural paintings are mostly colonised by filamentous fungi, yeasts and bacteria. Therefore, for this work were selected the predominant fungal strains: *Rhodotorula* sp., *Cladosporium* sp., *Aspergillus* sp., *Penicillium* sp. isolated from different mural paintings.

Enzymatic assays were performed in order to understand if these enzymatic systems are active in each microorganism culture, and if they can be used as biological markers to monitor biodegradation and biodeterioration. Therefore, the first approach intends to investigate if the enzymes arylsulphatase, β-glucosidase, phosphatase and dehydrogenase can be used for enzymatic monitorization of the microorganisms.

These enzymatic systems have been used in soils, water and wastewaters quality/contamination studies [17-25], being good indicators of biological activity, so this work intends to evaluate their potential in mural paintings degradation assisted or not by deterioration.

The results (Fig. 1) show that the enzymatic systems arylsulphatase, β-glucosidase, phosphatase and dehydrogenase are active in the fungal strains tested. However, β-glucosidase reveal less activity than the other biological systems.

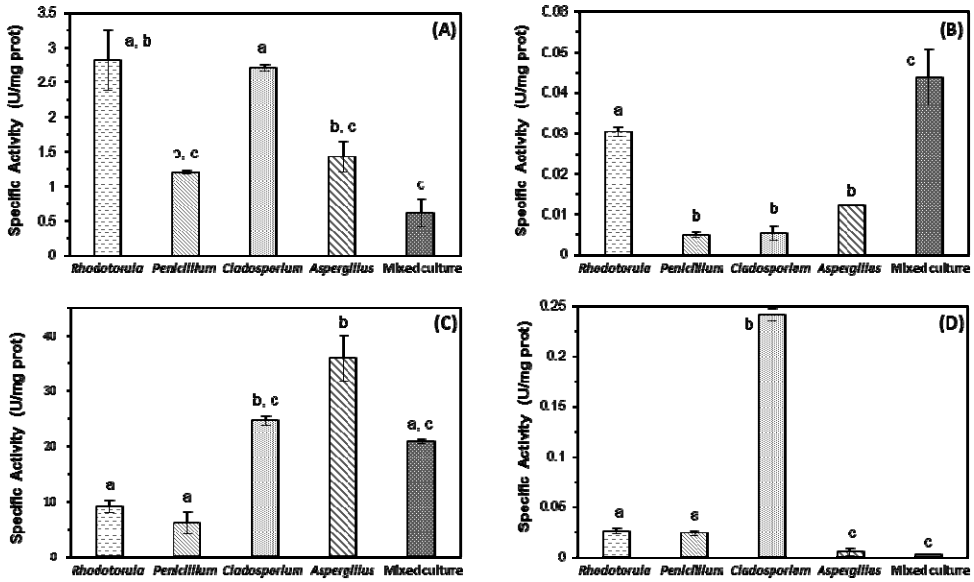

Fig. 1. Enzymatic monitorization of arylsulphatase (A), β-glucosidase (B), phosphatase (C) and dehydrogenase (D) in liquid cultures of predominant fungal strains isolated from mural paintings: *Rhodotorula* sp., *Cladosporium* sp., *Aspergillus* sp., *Penicillium* sp. and a mixed culture of these microorganisms. Different letters following the values indicate significant differences ($p < 0.05$). Values of each determination represents means ± SD (n = 3).

Arylsulphatase, β-glucosidase and phosphatase activity are correlated to the compounds transformations that provide different components to microorganisms development. In the case of dehydrogenase, their activity reflects the total oxidative activities of microorganisms and hence the presence of living cells [26], and can be used as an indicator of the presence of metabolic active cells.

Thus, the results obtained allow us to verify that all fungi tested have active enzymatic complexes studied, and suggest that these enzymes are good biochemical markers to evaluate the metabolic activity of the coloniser agents of mural paintings.

Simulation of the influence of these microorganisms on real sterilised mortars samples, inoculated with microorganisms, allows to verify its individual and combined action on the paintings, and, infer about their biodeteriogenic capacity, clarifying the fungal impact in the mural painting biodegradation and biodeterioration. In figure 2 it is possible to observe that the enzymes tested are active in these microorganisms on inoculated mortar samples, showing that it is possible to monitor enzymatic activity in mortars.

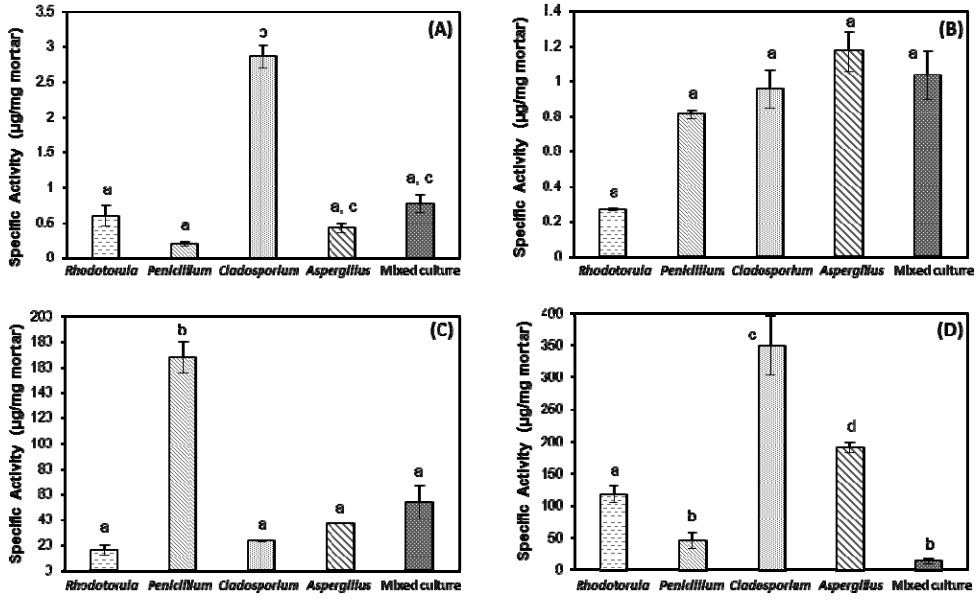

Fig. 2. Enzymatic monitorization of arylsulphatase (A), β-glucosidase (B), phosphatase (C) and dehydrogenase (D) in mortar simulated assays with the predominant fungal strains isolated from mural paintings: *Rhodotorula* sp., *Cladosporium* sp., *Aspergillus* sp., *Penicillium* sp. and a mixed culture of these microorganisms. Different letters following the values indicate significant differences ($p < 0.05$). Values of each determination represents means ± SD (n = 3).

Furthermore, in order to evaluate the effect of microbial proliferation in mural paintings and their impact in the degradation assisted or not by deterioration, real mortar samples with different visual alterations were analysed. These samples were named: Low Deteriorated Sites (LDS) and High Deteriorated Sites (HDS) according to the degradation levels observed.

Results show that samples from sites with larger signs of contamination present higher enzymatic activity (Fig. 3).

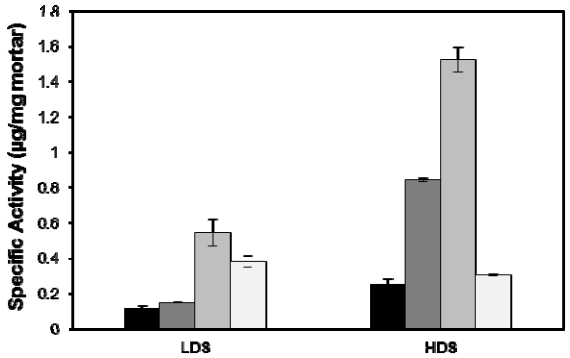

Fig. 3. Enzymatic assays to evaluate biological activity in samples with different contaminated levels, using dehydrogenase (■), arylsulphatase (▩), phosphatase (▣) and β-glucosidase (□) as biochemical markers. LDS – Low Deteriorated Sites; HDS – High Deteriorated Sites.

Assays in real mortar samples reveal that highest enzymatic activities were detected in samples from High Deteriorated Sites (Fig. 3), which is located in sites with greater degradation signals and concomitantly revealed high microbial colonisation, confirmed by SEM observation (Fig. 4). An exception in this behavior was obtained for the enzyme β-glucosidase that presented little variability. The results show that arylsulphatase, phosphatase and dehydrogenase can be used as biochemical markers in mural paintings, giving useful information about the biological activity of the microbial population, which can be correlated with the degradation status of the artwork.

The SEM analysis allowed a further insight on the presence of microbial communities thriving in the paintings and their capacity to proliferate within and/or penetrate inside the mortar structure. Results show that fungal proliferation conduces to penetration of mycelia structures in the microstructure of the mortars, promoting dissemination of these microorganisms in depth, whose behaviour seems to be correlated with cracks and the detachments observed in the painting (Fig. 4). Thus, fungal proliferation appears associated to mortar structural damages and chromatic alterations.

The microbial degradation assisted or not by deterioration effects on paintings can be caused due to the hydrolytic activities that the microorganisms undertake to sustain growth and, also due to the damage that excretion metabolites inflict [8]. Moreover, the production of extracellular polymeric substances (EPS), mainly polysaccharides, surrounding the hyphae, promotes the adhesion to the substrate leading to biofilm formation, as seen with SEM, that also contributes to the paintings degradation [27].

The results show that the combined approach using SEM analysis and metabolic activity measurement could be a useful methodology for the evaluation of microflora proliferation and the biodegradation and biodeterioration diagnosis of the mural paintings.

The results show that these enzymatic assays constitute good biomarkers for the biodegradation and biodeterioration assessment, giving a correlation with the degradation and deterioration levels of the paintings. However, enzymatic assays can be affected by several parameters like temperature, pH and substrate. Therefore, the methodology was refined by introducing a different approach based on the application of cell viability assays. This method is easier, quicker and provides good quality reproducible data with very small amount of sample.

These cell viability assays rely on the ability of living cells to reduce the MTT salt by respiratory chain and other electron transport systems/mitochondrial enzymes, in an insoluble purple formazan salt which can be quantified by spectrophotometry, giving information about the number of living cells in the sample. This approach has been used in immunology,

toxicology, and cellular biology, but never in cultural heritage biodegradation and biodeterioration studies [28, 29].

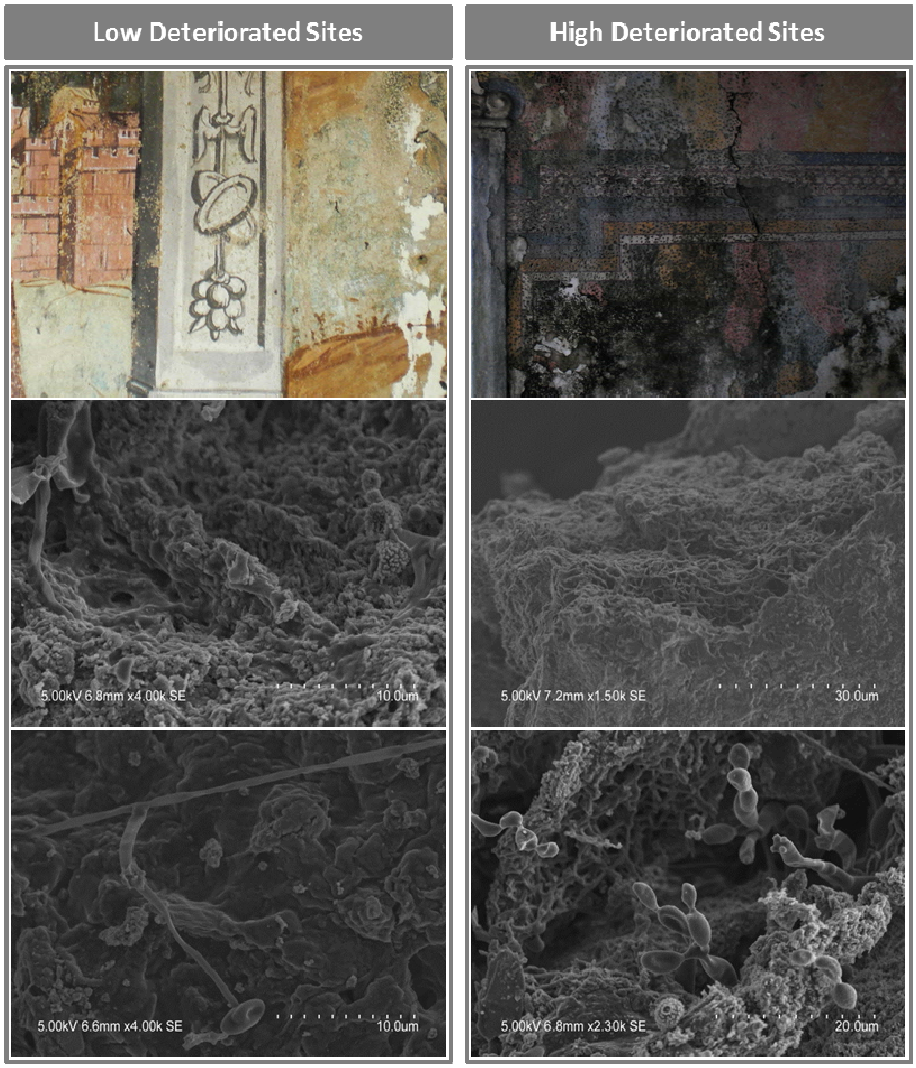

Fig. 4. SEM analysis of samples from Low Deteriorated Sites (LDS) and High Deteriorated Sites (HDS).

This assay was optimized to be applied in real mural paintings samples. The results are presented in figure 5 and show that samples from painting areas with low signals of degradation present low concentration of viable cells while samples from areas with evident signals of degradation have the opposite behaviour, *i.e.*, present high concentration of viable cells (Fig. 5). These results are according to the previous ones obtained by the enzymatic assays and this method is more advantageous because it is simple, fast and very sensitive.

Furthermore, to evaluate the method response were inoculated sterilised mortar samples with different concentrations of microorganisms. This assay presented a linear correlation with the microorganisms concentration, representing an optimal approach that can be used in mural paintings biodegradation and biodeterioration assessment to correlate degradation and

deterioration levels with metabolic active cells levels and consequently with microbial contamination degrees, constituting a very sensitive bioindicator.

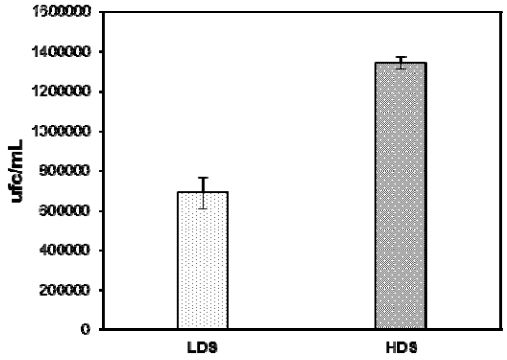

Fig. 5. Cellular viability of the microbial population present in mural paintings.
LDS – Low Deteriorated Sites; HDS – High Deteriorated Sites.

Conclusions

Enzymatic systems like arylsulphatase, β-glucosidase, dehydrogenase and phosphatase constitute good biomarkers to assess biological activity in mural paintings and can be correlated with the biodegradation and biodeterioration status of the artwork.

Cell viability assays based on mitochondrial enzymes (MTT assays) were optimized in this work to be applied in mortar samples and constitutes an efficient real time method to assess metabolic activity in microsamples, that allows infer about the active microbial contamination in the paintings.

These approaches are an important contribution to artworks biodegradation and biodeterioration research giving an overview of the hazardous effect of the microbial degradation and biodeteriogenic agents.

Acknowledgements

Tânia Rosado acknowledges Fundação para a Ciência e Tecnologia for financial support (PhD grant, SFRH/BD/65747/2009) through program QREN-POPH-typology 4.1., co-participated by the Social European Fund (FSE) and MCTES National Fund.

References

[1] D. Pangallo, K. Chovanová, H. Drahovska, F. De Leo, C. Urzì, *Application of fluorescence internal transcribed spacer-PCR (f-ITS) for the cluster analysis of bacteria isolated from air and deteriorated fresco surfaces*, **International Biodeterioration and Biodegradation**, **63**(7), 2009, pp. 868-872.

[2] S. Sequeira, E.J. Cabrita, M.F. Macedo, *Antifungals on paper conservation: An overview*, **International Biodeterioration and Biodegradation**, **74**, 2012, pp. 67-86.

[3] T.C. Dakal, S.S. Cameotra, *Microbially induced deterioration of architectural heritages: routes and mechanisms involved*, **Environmental Sciences Europe**, **24**(36), 2012, pp. 1-13.

[4] F. Cappitelli, P. Principi, C. Sorlini, *Biodeterioration of modern materials in contemporary collections: can biotechnology help?*, **Trends in Biotechnology**, **24**(8), 2006, pp. 350-354.

[5] G. Rolón, G. Cilla, *Adobe wall biodeterioration by the Centris muralis Burmeister bee (Insecta: Hymenoptera: Apidae) in a valuable colonial site, the Capayán ruins (La Rioja, Argentina)*, **International Biodeterioration and Biodegradation**, **66**(1), 2012, pp. 33-38.
[6] M.P. Nugari, M. Realini, A. Roccardi, *Cladosporium Contamination of mural paintings by indoor airborne fungal spores*, **Aerobiologia**, **9**, 1993, pp. 131-139.
[7] S. Borrego, P. Guiamet, S. Gómez de Saravia, P. Batistini, M. Garcia, P. Lavin, I. Perdomo, *The quality of air at archives and the biodeterioration of photographs*, **International Biodeterioration and Biodegradation**, **64**(2), 2010, pp. 139-145.
[8] A. Santos, A. Cerrada, S. García, M. San Andrés, C. Abrusci, D. Marquina, *Application of Molecular Techniques to the Elucidation of the Microbial Community Structure of Antique Paintings*, **Microbial Ecology**, **58**(4), 2009, pp. 692-702.
[9] M.I. Sarró, A.M. García, V.M. Rivalta, D.A. Moreno, I. Arroyo, *Biodeterioration of the Lions Fountain at the Alhambra Palace, Granada (Spain)*, **Building and Environment**, **41**(12), 2006, pp. 1811-1820.
[10] O. Pepe, S. Palomba, L. Sannino, G. Blaiotta, V. Ventorino, G. Moschetti, F. Villani, *Characterization in the archaeological excavation site of heterotrophic bacteria and fungi of deteriorated wall painting of Herculaneum in Italy*, **Journal of Environmental Biology**, **32**, 2011, pp. 241-250.
[11] P.W. Stege, G.A. Messina, G. Bianchi, R.A. Olsina, J. Raba, *Determination of arylsulphatase and phosphatase enzyme activities in soil using screen-printed electrodes modified with multi-walled carbon nanotubes*, **Soil Biology and Biochemistry**, **41**(12), 2009, pp. 2444-2452.
[12] J.P. Taylor, B. Wilson, M.S. Mills, R.G. Burns, *Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques*, **Soil Biology and Biochemistry**, **34**, 2002, pp. 387-401.
[13] M.A. Tabatabai, J.M. Bremmer, *Arylsulfatase of soils*, **Soil Science Society of America Journal, 34**, 1970, pp. 225–229.
[14] M.A. Tabatabai, J.A. Bremmer, *Use of p-nitrophenylphosphate for assay of soil phosphatase activity*, **Soil Biology and Biochemistry**, **1**, 1969, pp. 301–307.
[15] F. Camiña, C. Trasar-Cepeda, F. Gil-Sotres, C. Leirós, *Measurement of dehydrogenase activity in acid soils rich in organic matter*, **Soil Biology and Biochemistry**, **30**(8/9), 1998, pp. 1005-1011.
[16] T. Mosmann, *Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays*, **Journal of lmmunological Methods**, **65**, 1983, pp. 55-63.
[17] S.C. Antunes, R. Pereira, S.M. Marques, B.B. Castro, F. Gonçalves, *Impaired microbial activity caused by metal pollution: A field study in a deactivated uranium mining area*, **Science of the Total Environment**, **410-411**, 2011, pp. 87-95.
[18] D.W. Bergstrom, C.M. Monreal, A.D. Tomlin, J.J. Miller, *Interpretation of soil enzyme activities in a comparison of tillage practices along a topographic and textural gradient*, **Canadian Journal of Soil Science, 800**, 2000, pp. 71-79.
[19] C. Floch, Y. Capowiez, S. Criquet, *Enzyme activities in apple orchard agroecosystems: How are they affected by management strategy and soil properties*, **Soil Biology and Biochemistry**, **41**(1), 2009, pp. 61-68.
[20] R. García-Ruiz, V. Ochoa, B. Viñegla, M.B. Hinojosa, R. Peña-Santiago, G. Liébanas, J.C. Linares, J.A. Carreira, *Soil enzymes, nematode community and selected physico-chemical properties as soil quality indicators in organic and conventional olive oil farming: Influence of seasonality and site features*, **Applied Soil Ecology**, **41**(3), 2009, pp. 305-314.
[21] E. Jastrzębska, J. Kucharski, *Dehydrogenases, urease and phosphatases activities of soil contaminated with fungicides*, **Plant Soil and Environment**, **53**(2), 2007, pp. 51-57.

[22] H. Kang, S. Kang, D. Lee, *Variations of soil enzyme activities in a temperate forest soil*, **Ecological Research**, **24**(5), 2009, pp. 1137-1143.
[23] S. Klose, H.A. Ajwa, *Enzyme activities in agricultural soils fumigated with methyl bromide alternatives*, **Soil Biology and Biochemistry**, **36**(10), 2004, pp. 1625-1635.
[24] C. Pozo, M.V. Martínez-Toledo, B. Rodelas, J. González-López, *Response of soil microbiota to the addition of 3,3'-diaminobenzidine*, **Applied Soil Ecology**, **23**(2), 2003, pp. 119-126.
[25] F. Balestri, R. Moschini, M. Cappiello, A. Del-Corso, U. Mura, *Impact on enzyme activity as a new quality index of wastewater*, **Journal of Environmental Management**, **117**, 2013, pp. 76-84.
[26] L. Huang, X. Gao, M. Liu, G. Du, J. Guo, T. Ntakirutimana, *Correlation among soil microorganisms, soil enzyme activities, and removal rates of pollutants in three constructed wetlands purifying micro-polluted river water*, **Ecological Engineering**, **46**, 2012, pp. 98-106.
[27] L. Zucconi, M. Gagliardi, D. Isola, S. Onofri, M.C. Andaloro, C. Pelosi, P. Pogliani, L. Selbmann, *Biodeterioration agents dwelling in or on the wall paintings of the Holy Saviour's cave (Vallerano, Italy)*, **International Biodeterioration and Biodegradation**, **70**, 2012, pp. 40-46.
[28] F.M. Freimoser, C.A. Jakob, M. Aebi, U. Tuor, *The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] Assay Is a Fast and Reliable Method for Colorimetric Determination of Fungal Cell Densities*, **Applied and Environmental Microbiology**, **65**(8), 1999, pp. 3727-3729.
[29] I. Mota, P.C. Rodrigues Pinto, C. Novo, G. Sousa, O. Guerreiro, Â.R. Guerra, M.F. Duarte, A.E. Rodrigues, *Extraction of Polyphenolic Compounds from Eucalyptus globulus Bark: Process Optimization and Screening for Biological Activity*, **Industrial and Engineering Chemistry Research**, **51**(20), 2012, pp. 6991-7000.

Received: October, 11, 2013
Accepted: December, 10, 2013