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RESEARCH ARTICLE

Understanding the influence of microbial contamination on colour alteration of pigments used in wall paintings—The case of red and yellow ochres and ultramarine blue

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Abstract

This article reports biocolonization assays carried out on yellow and red ochres and ultramarine blue pigments. These pigments have been most commonly used in traditional limewash of heritage buildings from southern Portugal (Alentejo) and, in the case of the yellow and red ochres, also in historical decorative wall paintings since Roman times. The research aim was to assess the potential role played by microorganisms in colour alterations observed in indoor and outdoor paint layers for conservation purposes.The assays accomplished several microorganisms previously isolated from degraded wall paintings with signs of biocontamination. The results show that apart from the clear physical stress induced in paint layers by the biometabolic activity, filamentous fungi, yeast, and bacteria are capable of inducing discoloration (in particular, the fungus *Aspergillus niger*). Raman analysis corroborates their active role in painting discolouration. This methodology, applied to bioprocesses, can be used as noninvasive methodology to signal microbial involvement.

KEYWORDS

biodeterioration, chromatic alteration, microbiological proliferation, mural painting, Raman analyses

1 | INTRODUCTION

Yellow and red ochres and artificial ultramarine blue pigment were, and still are, the traditional and standard colours of Alentejo (southern Portugal) Historic Building Heritage. These pigments were widely used in the past by the local populations in limewash outdoor paintings (façades) and, in the case of the ochres, also for centuries by artists in outdoor and indoor decorative wall paintings with *fresco* and *secco* techniques.[1-5]

Wall paintings are some of the oldest and most important cultural expressions of mankind and play an important role in understanding societies and civilizations. These assets have high economic and cultural values and therefore, their degradation is a problem with social and economic impact, which must urgently be addressed for their future preservation.[6]

From a chemical point of view, yellow and red ochres are earth pigments, that is, natural clay-based materials. Their colours depend on the presence and nature of the iron hydroxides and oxides chromophores, especially goethite and hematite.[7-9] The geological sources may vary, as different shades occur in many surface deposits (eg, schists, iron ores, carbonate rocks). Portugal is rich in raw materials, which were, and can still be, used.[6,7,9] Due to their availability and ease of extraction and preparation, natural ochres

were widely used by the population in Alentejo country side until the first half of the 20th century. On the other hand, professional painters seem to have always preferred the industrial processed analogues (industrial ochres), iron oxides, and the synthetic ultramarine blues. Traditionally, the blue colour has been more associated to towns of south Alentejo (near Algarve and the Mediterranean). In the case of the synthetic blue pigment, lazurite is the major component to which other minerals can be added as fillers.[3]

All these pigments, in particular earth pigments, are inorganic and usually show physical and chemical stability. However, under aggressive weathering conditions, colour alteration can occur. High humidity content, temperature, light, and atmospheric pollution are among the main parameters that strongly influence the decay of mural paintings.[6,7,10-17] In some cases, colour alterations in paint layers are detected in the areas showing signs of biocontamination. Several investigations have shown that both environmental factors and microbial contamination can contribute to pigment alteration,[18-21] but little is still known about the interaction itself and how deep microorganisms can be the main promoters of paint colour decay. Regarding this, knowledge about the interaction of the microbial population with the physicochemical properties of the materials is considered central to the understanding of the long-term degradation/deterioration of indoor and outdoor mural paintings.[22-24]

In this study, different microorganisms previously isolated from altered mural paintings with signs of biocontamination, inserted in distinct environments, were selected to assess the possible biogenic involvement in the chromatic alteration processes that affect ochres and blue pigments.

2 | EXPERIMENTAL

To evaluate the influence of microbiological colonization on red and yellow ochres and synthetic ultramarine blue, bacterial and fungal isolates, previously isolated from altered wall paint layers, were selected.

2.1 | In vitro simulation assays

To assess the role of the microorganisms in the pigment alteration, simulation assays were performed in liquid culture, in the presence of yellow and red ochre and synthetic ultramarine blue pigments. Four bacterial isolates (*Arthrobacter* sp.1-CCLBH-BP301, *Bacillus* sp.1-CCLBH-BP102, *Arthrobacter* sp.2-CCLBH-BP302, and *Bacillus* sp.2-CCLBH-BP103) and four fungal isolates (*Cladosporium* sp.-CCLBH-MP602, *Penicillium* sp.-CCLBH-MP102, *Aspergillus niger*-CCLBH-MP202, and *Rhodotorula* sp.-CCLBH-YMP502), belonging to the culture collection of HERCULES-Biotech Laboratory (CCLBH) of Évora University, were used as biological models.

Fresh bacterial and fungal cultures were prepared in solid nutrient agar and malt extract agar media, respectively. Cell suspensions (10^8 CFU) were prepared by washing each slant with NaCl 0.85% solution. Each microbe isolated was inoculated in the presence/absence of each pigment (previously sterilized), in 100 mL of nutrient broth (for bacteria) and malt extract (for fungi) and incubated at 30°C and 28°C, respectively, at 120 rpm (IKA KS 4000 ic control). Samples were collected in sterile conditions at 0, 2, 4, 7, 11, 14, 16, 21, 24, and 31 days.

2.2 | Monitoring of simulation assays

2.2.1 | Colour monitoring and microbial development

Samples (200 μL) from each liquid assay were collected periodically, as described in Section 2.1, and spectrophotometrically monitored at 200 to 900 nm on the Multiskan Go Microplate Spectrophotometer (Thermo Scientific).

2.2.2 | Quantification of chromatic alteration

Colour alteration of each simulation assay was also measured by a noninvasive spectrophotometer Datacolor CheckPlus II (DataColor, Lawrenceville, New Jersey) equipped with an integrating sphere, in the following conditions: illumination 8° viewing (in agreement with the International Commission on Illumination [CIE] publication No. 15.2. Colorimetry), SCE, and Standard Illuminant/Observer D65/10°. The aperture size used was (5 mm). The results obtained in the CIE $L^*a^*b^*$ chromatic space defined by the CIE in 1976 represent the average of three measurements taken on the paint layer surface. The chromatic coordinates measured were L^*, which represents lightness (0-100); a^*, which stands for the red/green axes; and b^*, which stands for the yellow/blue hue axes (0-100). The C^* coordinate, derived from the previous colour space (CIE $L^*C^*h^*$), which represents the chroma (colour purity or saturation), was also analyzed.[5]

2.2.3 | Cellular viability assessment

Cell viability of the microorganisms tested in the presence of yellow and red ochre and ultramarine blue pigments was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described by Mosmann[25] and applied and optimized by our investigation group.[26]

Aliquots (100 μL) of each assay were collected and incubated with 300 μL of MTT stock solution (5 mg/mL in Phosphate-Buffered Saline 1x), for 4 hours, in the dark, at 37°C. After this period, 350 μL of Dimethyl sulfoxide/ethanol (1:1) was added to dissolve the formazan crystals formed. The final suspension was centrifuged at 10 000 rpm for

15 minutes and the supernatant was spectrophotometrically (Hitachi, U-3010) analyzed at 570 nm. Each assay was performed in triplicate.

2.2.4 | Detection of pigments alteration

Raman spectra were acquired using a HORIBA Xplora Raman microscope coupled to external power laser sources for specimen radiation: 638 nm (He-Ne). Irradiation of samples was performed using a filter 10% to 50% to prevent any thermal damage of the material. Ordinary acquisition time was on the order of 10 to 20 seconds with 5 cm^{-1} of spectral resolution. The backscattered light was collected by the objective (×10 or ×50), and then captured by a charge-coupled device detector. Spectra were calibrated using the 520.5 cm^{-1} band of a silicon wafer. Spectra deconvolution was performed using LabSpec, and the identification was made with Spectral IDTM 13.

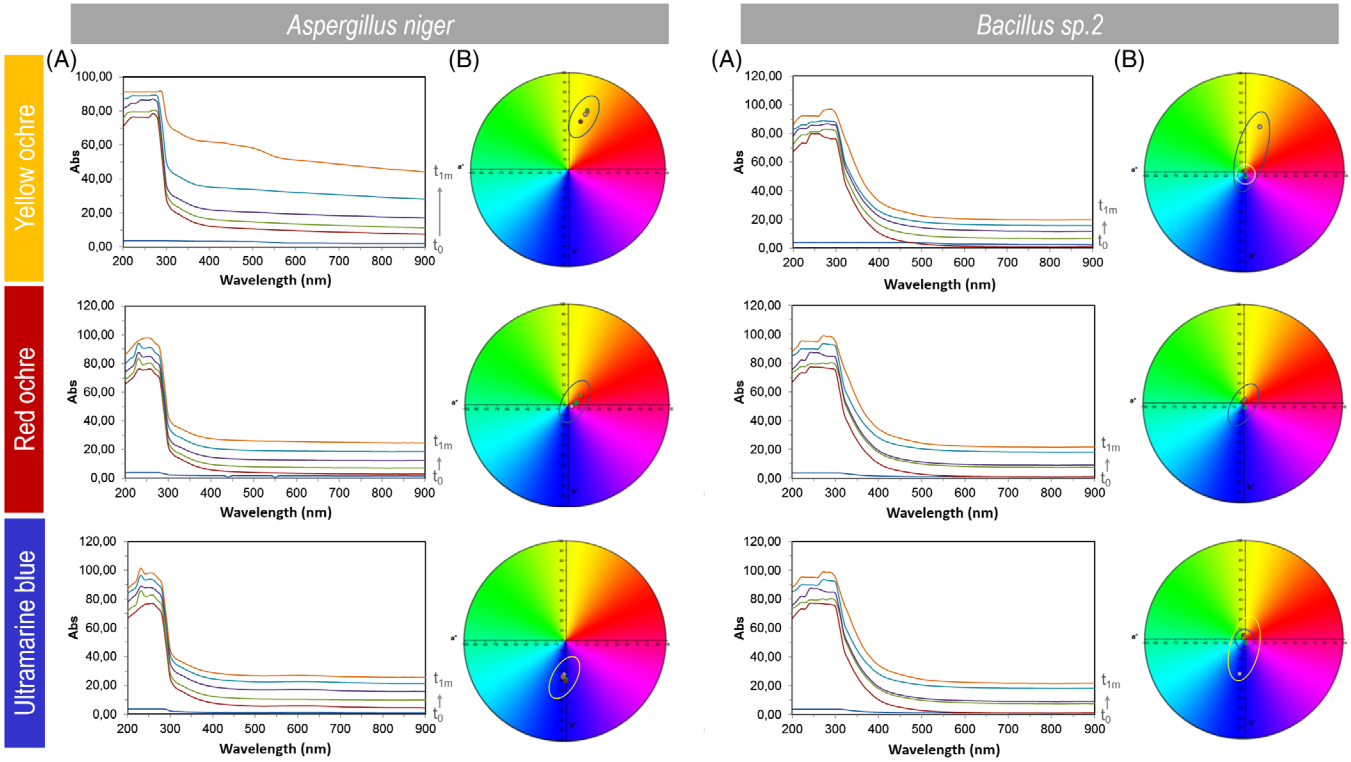

FIGURE 1 Color evaluation of yellow red and blue pigments, by A, UV-Vis spectrophotometry and B, CIELAB colour space, inoculated with *Aspergillus niger* and *Bacillus* sp.2

TABLE 1 Variation of the colorimetric parameters (ΔE represents the differences calculated in beginning and in the end of the assays for each pigment and microorganism) in the CIELAB colour space

		Chromatic parameters			
Microorganism	**Pigment**	**ΔL^***	**Δa^***	**Δb^***	**ΔE^***
Bacillus sp.2	YO	1.32	1.66	2.59	3.35
	RO	−14.14	8.24	10.95	19.69
	SUB	−8.12	−0.92	−1.83	8.37
Aspergillus niger	YO	21.54	−14.07	−43.76	50.76
	RO	2.9	−1.36	−0.75	3.29
	SUB	15.8	5.68	38.07	41.61

$\Delta E^* = \sqrt{(\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})}$.

Abbreviations: RO, red ochre; SUB, synthetic ultramarine blue; YO, yellow ochre.

3 | RESULTS AND DISCUSSION

Standard tests containing the pigments yellow, red, and blue, as well as simulation assays containing each of these pigments in the presence of the selected microorganisms, were performed in order to evaluate whether the chromatic alterations can be induced by biogenic involvement.

The analysis performed allowed to follow up the microbial growth and to screen the pigment spectra along the simulation assays.

Figure 1 shows the time scan (Figure 1A) and the colour evaluation on the CIELAB space (Figure 1B) of the yellow, red, and blue pigments in the presence of the filamentous fungus *Aspergillus niger* and the strain of bacteria *Bacillus* sp.2.

The spectrophotometric analysis revealed that these microorganisms possess ability to promote pigment alteration in culture.[27] However, the bacterial strains (particularly *Bacillus* sp.2) tested induce less colour alterations than the fungi and show higher alteration capacity for yellow

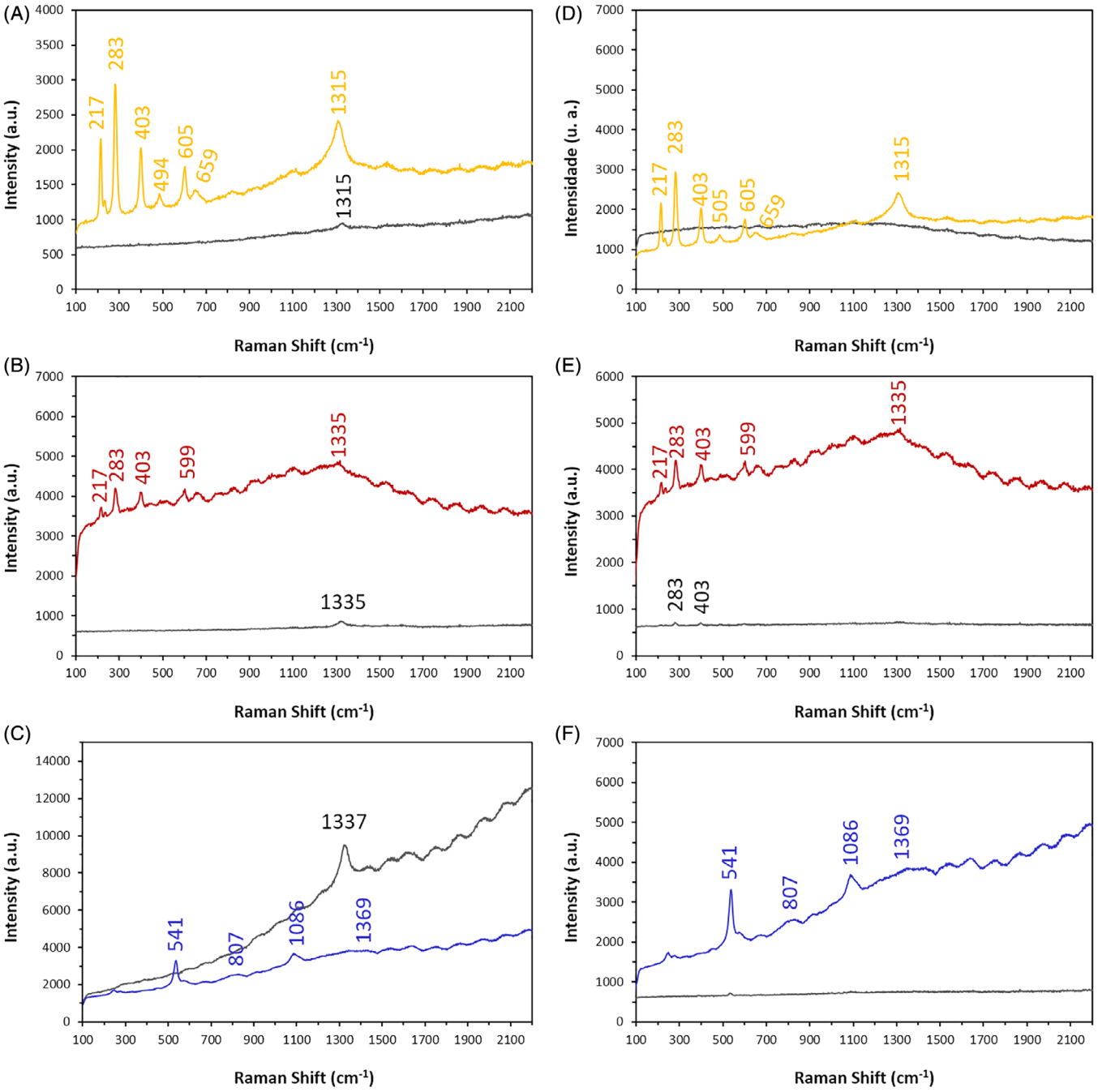

FIGURE 2 Raman spectra of simulation assays performed with yellow, red, and blue pigments inoculated with A-C, *A. niger* and D-F, *Bacillus* sp.2 (——: yellow ochre standard pigment; ——: red ochre standard pigment; ——: ultramarine blue standard pigment; ——: pigment inoculated with microorganism)

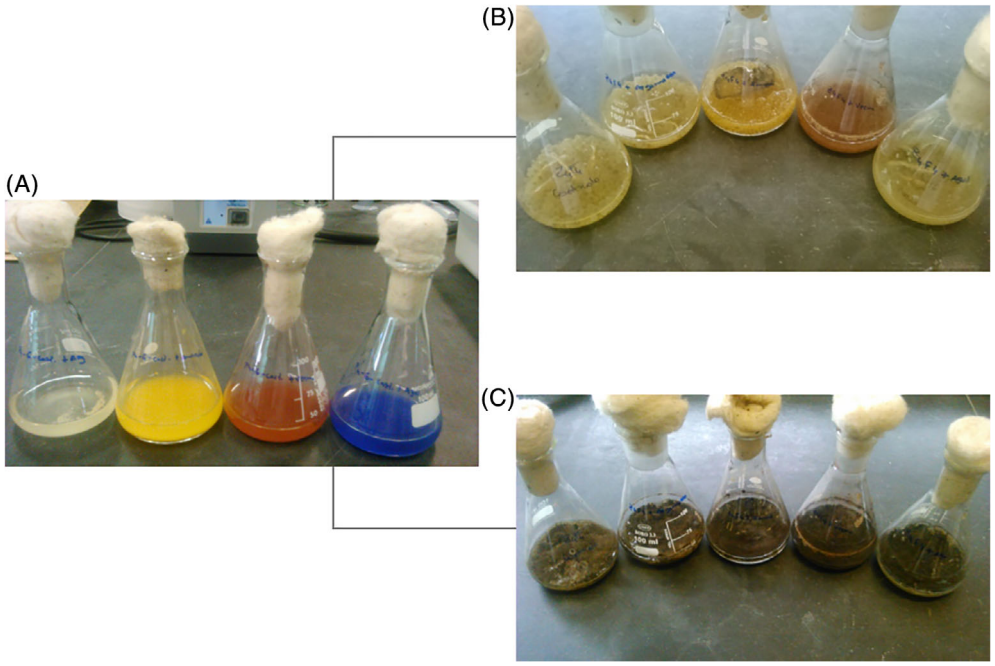

FIGURE 3 Liquid simulation assays performed with A, yellow, red, and blue standard pigments and the same pigments inoculated with B, *Bacillus* sp.2 and C, *A. niger*

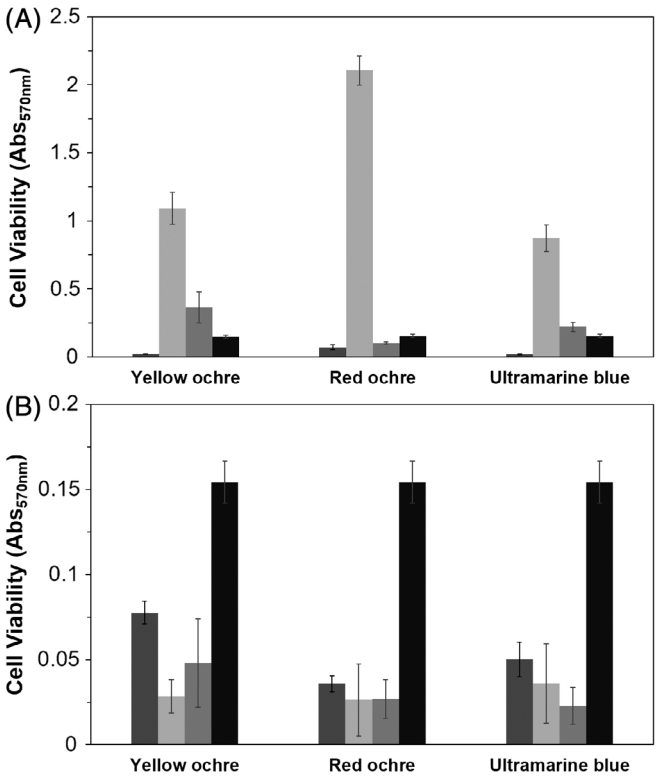

FIGURE 4 Cell viability assessment of A, *Bacillus* sp.2 and B, *A. niger* in the presence of yellow, red, and blue pigments (■: t_{0d}; ■: t_{2d}; ■: t_{11d}; ■: t_{30d})

pigments. This behavior was more evident with *A. niger*, which induced total discolouration of all the pigments, as shown by the CIELAB system (Figure 1 and Table 1).

To complement the spectral characterization, Raman spectrometry was also used.

All the simulation assays mentioned above were analyzed by Raman, results of which indicate that these microorganisms affect the pigment signals, making all the characteristic peaks of each pigment disappear, as shown in Figure 2.[28]

Thus, these results evidenced that pigments like yellow ochre, red ochre, and ultramarine blue suffered total and/or partial discolouration after 30 days in the presence of fungi and bacteria, as can be visually observed in Figure 3.

Aspergillus niger revealed high biodeteriorative capacity, having the ability to incorporate the pigments. The results show the ability of microorganisms to proliferate and induce aesthetic alteration on the pigments used in mural paintings by chromatic alterations. Thus it is necessary to understand if the pigments affect the normal development of the biocolonizers. In this way, metabolic assays to monitor the viability of the cells in the presence of the yellow, red, and blue pigments were assessed (Figure 4). These microorganisms revealed the ability to proliferate at high concentrations of these pigments, remaining metabolically active even after 30 days in contact with these pigments, which evidences their potential capacity to colonize and to induce alteration on mural paintings.[27,29,30]

Cell viability assays corroborate the indications obtained by colorimetric and spectral monitorization, where the chromatic degradability of fungi and bacteria was confirmed, as well as their ability to proliferate in the presence of these pigments, whose metabolic activity remains active even under reduced nutritional conditions.

4 | CONCLUSIONS

This work shows that filamentous fungi, yeast, and bacteria have the capacity to degrade and/or induce chromatic

alterations on yellow and red ochres and ultramarine blue pigments widely used in mural paintings and traditional limewash, which seems to be related to some aesthetic damages that affect these artworks.

Aspergillus niger promoted high discolouration of the pigments, evidencing their biodeteriorative ability.

Fungi and bacteria revealed their ability to proliferate at high concentrations of pigments, remaining metabolically active even under reduced nutritional conditions, which proves their capacity to colonize and damage these cultural heritage assets.

The current work is a step forward to the understanding of biodegradation of pigments; however, further studies are needed to explore the complete mechanism and pathways involved in the microbial degradation of mural paintings. Furthermore, the high tolerance and degradative capacity of some microorganisms to degrade pigments suggest their applicability and potential in transversal areas of knowledge.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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Ana Falé received a degree in biochemistry from the University of Évora, Portugal, in 2016. Her investigation work focused on the biodegradation of mural paintings in several cultural heritage assets. Currently, she is pursuing a master's degree in technology and food safety at the Faculty of Sciences and Technologies of the Universidade Nova de Lisboa.

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José Mirão received a PhD degree in geology from the University of Évora, Portugal, in 2004. His research interests include the study of heritage (eg, ceramics, lithics, alloys, glass) and geological materials (eg, ores, soils) using microprobe and in situ analytical tools. He is a senior researcher in HERCULES Laboratory and a professor in the Geosciences Department of the University of Évora.

António Candeias specialized in chemistry applied to cultural heritage and surface chemistry. He is a professor in the Chemistry Department of the University of Évora, Portugal, and the director of HERCULES Laboratory. In terms of research activity, his main interests have focused on the study of mortars and renders, study and characterization of cultural assets and archeological materials and optimization and application of analytical techniques for the study of cultural heritage, with special emphasis on microanalysis and in situ analysis.

Ana Teresa Caldeira is a professor at the Department of Chemistry at the University of Évora, Portugal, specialized in biochemical analysis and microbial physiology. She is the coordinator of the Biotechnology and Biodegradation Unit of HERCULES Lab. Her main areas of research focus on new strategies for diagnosis, monitoring, and mitigation applied to cultural heritage including new methods for measuring biodegradation, development of new green biocides, and methodologies for the identification of biomolecules.

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