Characterization of microbial colonization of three mural painting's churches (N. Portugal): Preliminary study of microbial growth, biocides activity evaluation and future intervention proposal

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Introduction

The research comes to a focus on three case studies: Santa Eulália Church of the Monastery of Arnoso (Vila Nova de Famalicão); São Tiago of Valadares Church (Baião); and Santa Cristina of Serzedelo Church (Guimarães).
The three Romanic churches, made of porphyritic granite (biotitic) with coarse grain, fall within rural environments as perceived by the consultation of geological maps. Santa Eulália is from the XIIth century, Santa Cristina of the middle of the XIIIth century, and São Tiago probably of the end of the XIIIth century. The interior walls are partially covered with mural paintings: Sta. Eulália presents a first campaign possibly from the XVth century and overlaying it, one from the XVIth century. Sta. Cristina has several campaigns on different walls, being the latest from the XVIth century. S. Tiago has only one campaign from the XVth century.

Objectives

The present study relates to an investigation around three sets of fresco mural paintings showing surface alteration due to microbial colonization. The aim was both to document the procedures for collecting and processing samples in order to gather a wide range of knowledge of the original mural painting technique, and to characterize the current state of heritage conservation so as to plan conservation and restoration measures.

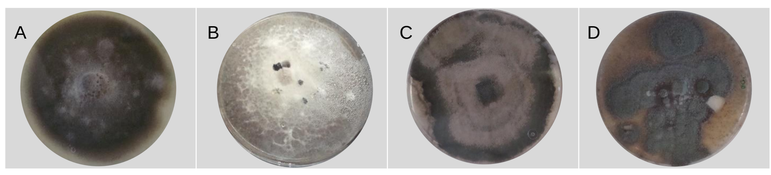

Fig. 3. Fungal strains tested in biocides assays: (A) Fungi 1; (B) Fungi 2; (C) Fungi 3; (D) Fungi 4.

Materials and Methods

Twenty seven samples were collected during the investigation and underwent analysis to identify the constitution of the mural paintings by using µS-FTIR combined with stratigraphic analysis **(Fig. 1)**.
The study of the surface changes unveiled a degradation, common to the three monuments, shown by the presence of dark biofilms of microbiological origin. Different methods of exam were undertaken for their study and characterization assessment **(Fig. 2)**: optical microscopy; classical microbiology methods and isolate identification by molecular biology; scanning electron microscopy (SEM); CIE L*a*b*; and spectrometry of UV-Visible.
Based on thirty six samples taken from the location where the chromatic changes were visible, twenty six filamentous fungi and forty three bacteria/yeasts were isolated. Seven filamentous fungi were identified to the species.
Four fungi isolates were selected among those with the capability of producing black pigmentation **(Fig. 3)** for treatments with four commercial biocides [CaSoPaL® (IBZ), Durolith® Bioclean L (Kremer), Benzalkonium Chloride (Antares) and Preventol R80 (Agar Agar)] and five essential oils [rosemary (*Rosmarinus officinalis*), fennel (*Foeniculum vulgare*), lemon (*Citrus limon*), basil (*Ocimum basilicum*) and sage (*Salvia officinalis*)], and the minimum inhibitory concentrations (MIC) determined **(Table 1)**. Assays were implemented for the pigment chemical isolation and characterization produced by these fungi and for the removal of the patina through pigment extraction protocols and fungal peroxidase enzymatic degradation and tested with a fungal versatile peroxidase from *Bjerkandera adusta* (JenaBios) **(Fig. 4; Fig. 5; Table 2; Fig. 6).**

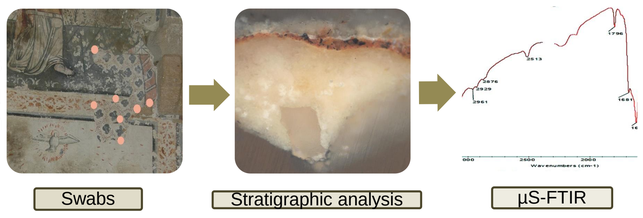

Fig. 1. Process of mural painting's characterization, from swab sampling to stratigraphic and Fourier transform infrared micro-spectroscopy analysis.

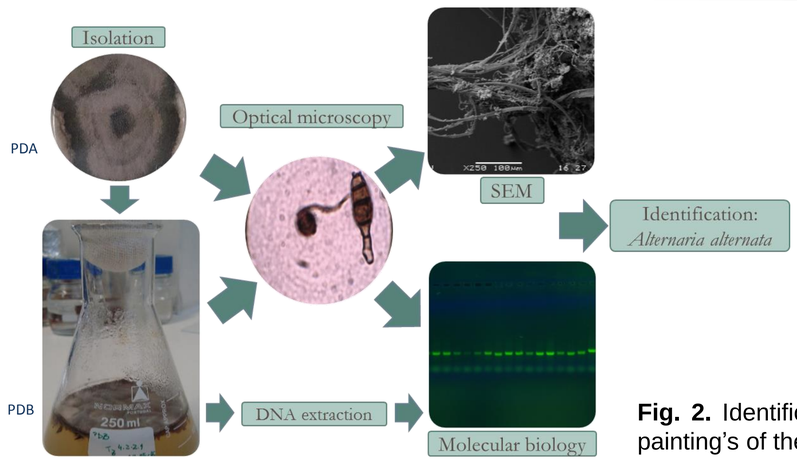

Fig. 2. Identification of isolates from mural painting's of the three churches.

Table 1. Minimum inhibitory concentration (MIC) of Preventol R80, Benzalkonium Chloride and essential oil *O. Basilicum* in selected fungal isolates.

Fungi	Preventol R80	Benzallkonium Chloride	Essential oil *Ocimum basilicum*
	MIC (µl·ml^{-1})	MIC (µl·ml^{-1})	MIC (µl·ml^{-1})
Fungi 1 (*Alternaria tenuíssima*)	n.d.	3,125	1,25
Fungi 2	12,5	1,563	0,625
Fungi 4 (*Alternaria alternata*)	6,25	6,25	1,25
Fungi 4	3,125	3,125	1,25

Legend: n.d. – not determined. Fungi 2 under identification, potentially *Pestaloptia sp*. Fungi 4 under identification, potentially *Penicillium sp*.

Fig. 4. Filamentous fungal isolate $CZ_{1.2.3}$ displaying colourless hyphae and production of black exudates and deep extracellular pigmentation from a sample collected from indoor of St. Cristina Church. (A) Solid medium. (B) Liquid medium.

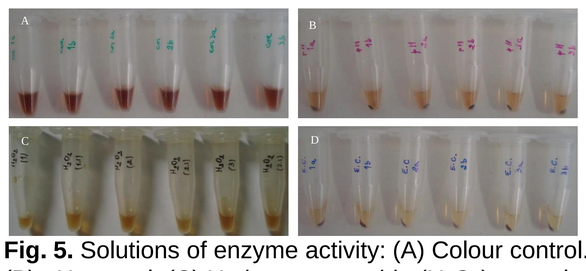

Fig. 5. Solutions of enzyme activity: (A) Colour control. (B) pH control. (C) Hydrogen peroxide (H_2O_2) control. (D) full assay.

Table 2. Measurement of total colour difference (ΔE) in sample papers using the 1976 CIELAB formula: Δ*E**ab = $[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{½}$

Colour difference (ΔE)	Incubation temperature	
	20ºC	30ºC
Δ1 - Δ0	8,2084	9,0221
Δ2 - Δ1	3,7753	3,0872
Δ3 - Δ1	4,3299	4,7822
Δ4 - Δ1	1,1546	2,0568
Δ4 - Δ0	7.0731	7.0302

Δ0 - Control. Δ1 - Colour. Δ2 - pH. Δ3 - H_2O_2. Δ4 - Complete assay.

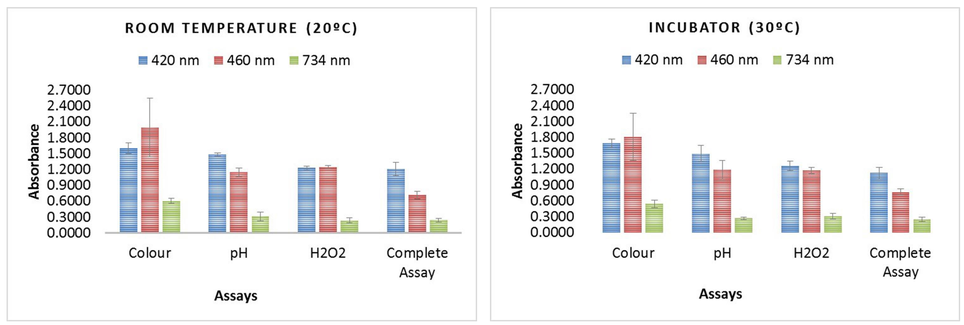

Fig. 6. Absorbance at 420, 460 and 734 nm measured for the supernatants from the enzymatic activity assays.

Conclusions

- ❑ Mural painting of all three churches revealed to be frescos, showing mostly just one layer of mortar and extremely thin layers of plaster and polycromy;
- ❑ Seven isolates were identified by molecular biology through ITS amplification and belonged to three different genera – *Aspergillus*, *Penicillium* and *Alternaria*;
- ❑ Among the nine biocides tested, the one that showed highest efficacy for all fungi was the basil essential oil. The mycelial growth of the 4 fungi was completely inhibited at 1,25 µl·ml^{-1} of this essential oil per plate [in Potato Dextrose Agar (PDA, Lab M)];
- ❑The results for pigment discolouration in mural paintings were promising regarding the application of hydrogen peroxide (Sigma) and fungal versatile peroxidase *Bjerkandera adusta* achieved *in vitro* on a total volume solution of 167,5 µl. The H_2O_2 control used 15 µl H_2O_2 at 30% + 50 µl pigment suspension + 102,5 µl H_2O. The complete assay used 2,5 µl enzyme + 15 µl H_2O_2 at 30% + 100 mM pH 3,5 Na-Ta buffer + 50 µl pigment suspension.

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