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Accelerated laboratory test to study fungal biodeterioration of cementitious matrix

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Keywords:
Biodeterioration ; fungi; cement ; accelerated laboratory test ; PAS staining.

Paper relevance;

The relevance of this paper is first the combination of the methods used for accelerated weathering of the matrix which leads to an important surface pH decrease in 30 days. Then, the originality of this paper is that the test presented necessitates only three months of experiment to obtain results, which is shorter than other test developed to date to study fungal biodeterioration.

Abstract

The present study aimed to develop an accelerated laboratory test to study the biodeteriorative effect of different fungal strains to a cementitious matrix. The test developed in this study permits to obtain a rapid fungal development on cement specimens. Three months of experiments only are needed to obtain first results, which is rather shorter than other test developed to date to study fungal biodeterioration. Results are mainly related to aesthetical biodeterioration. Results show that in these experimental conditions, fungal growth occurs since the first week of incubation. Stereomicroscopy observations showed that microbial growth was noticed only on the surface of specimens, while PAS staining revealed the real extent of microbial growth on and within the matrix as later confirmed by SEM observations of cross section showing the penetration of hyphae inside the matrix. Test can be used with short time of incubation, to test and to compare bioreceptivity of cement based materials; and several months of incubation should allow the study of mechanisms involved in biodeterioration

1. Introduction

Microorganisms – bacteria, cyanobacteria, fungi, algae, and lichens – are liable to grow on building materials. Biological activity contributes to deterioration of building material, and its interaction with physico-chemical mechanisms is considered central to understanding the long term deterioration (Saiz-Jimenez, 1997). Physical, chemical, and biological agents act in co-association, ranging from synergistic to antagonistic, to deteriorate stone (Warscheid and Braams, 2000).

Fungi are among the most harmful organisms associated to biodeterioration of organic and inorganic materials (Urzì et al., 2000a,b). Their occurrence on the stones is reported to be combined not only with aesthetic spoiling of the monuments, due to colour changes such as patina or black spots, but also there is strong evidence that these organisms are able to colonize deeper cracks, cause crater shaped lesions, chipping and exfoliation of the rock surface combined with the loss of materials (Wollenzien et al., 1995; Urzì et al., 2000b). May et al. (1993) listed fungi among major agents of microbial deterioration of building stones. Mineral substrates exposed to environmental stresses such as wind erosion, direct rain wash off, lack of organic nutrients and are refractory to rapid mycelial growth. To overcome these limitations, some fungal groups have abandoned their branched hyphal elongation system (which is more appropriate to the penetration and utilization of substrates) and have adopted microcolonial or yeast-like growth (Wollenzien et al., 1995). The smaller, compact shapes are more thermodynamically efficient especially in terms of protecting against heat and desiccation (Gorbushina, 2007).

In general, two main groups of fungi are usually isolated from rock surfaces (De Leo and Urzì, 2003): (i) one group includes species of the genera of *Hyphomycetes* and *Coelomycetes* among which are included those that do not produce melanin, like *Fusarium*, *Penicillium*, *Aspergillus*, and those that are black pigmented like *Alternaria*, *Ulocladium*, *Cladosporium* melanin producers. They are fast growing fungi. (ii) The second group includes the so-called black yeasts and meristematic fungi. Taxonomically, they are a wide and heterogeneous group of black pigmented fungi that share common characteristics such as the presence of melanins within the cells (swollen cells), hyphae and/or spores. The production of melanin and the meristematic development allow them to survive in stressed environmental conditions like low humidity and high sun irradiation (de Hoog, 1993). For this group of fungi is also used the term of rock-inhabiting fungi to underline the exclusive isolation of many of them from rock surface (De Leo and Urzì, 2003). The black fungi have the capacity to: (i) settle on the rocks surface, (ii) attach firmly to the surface and (iii) penetrate deeper into the rock. The major aesthetic damage, however, may occur when environmental conditions do not force fungi into the crevices, but due to favourable conditions for fungal growth they spread over the rock surface (Diakumaku et al., 1995).

In Civil Engineering the most widely used material is concrete for different kind of application: bridge construction, sewer pipes, buildings and in some case during restoration of Cultural Heritage Monuments. Cement plays an essential role in concrete works behaviour, because it provides its mechanical resistance (Guillon, 2004).

Cement based materials are porous, may contain organic adjuvants, and thus possess an important primary bioreceptivity. Bioreceptivity, as defined by Guillitte (1995), is the totality of materials properties that contribute to the establishment, anchorage and development of fauna and/or flora. Primary bioreceptivity is the initial potential of colonisation (Guillitte, 1995).

To preserve constructions from fungal colonisation and to act efficiently against fungal biodeterioration, it is necessary to have a better understanding of biodeterioration mechanisms and its effects on materials properties. At the moment, tests to study biodeterioration of building materials exist. Among them some were developed without accelerated weathering of the matrix leading to experiment time ranging from 7 to 15 months (Oshima et al. 1999; Urzì and De Leo 2007), while some focus on qualifying aesthetic evolution of external wall surface exposed to algal colonisation (Escadeillas et al. 2007), or studying the matrix bioreceptivity only (Nielsen et al. 2004; Shirakawa et al. 2003). To our knowledge, to date, only de Moraes Pinheiro and Ribas Silva (2003) studied the impact of

colonisation by one fungal strain on the microstructure in the mortar phase of a normal concrete.

The aim of this study is to develop an accelerated laboratory test which allows us to compare the growth of three fungal strains and the biodeterioration of a cementitious matrix. We brought particular attention to accelerate the matrix weathering so as to not exceeding 3 months of experiment. Three fungal strains were selected for the test in order to represent main kind of fungi involved in biodeterioration in natural environment (de la Torre et al., 1991; Sterflingler 1995; Wollenzien et al., 1995; Garcia Valles et al., 1997; Warscheid and Braams, 2000; Urzì et al., 1998, 2001): *Alternaria alternata* MC342 to represent an hyphomycete, melanin-producer *Exophiala* sp. (MC843) for yeast-like fungi, and *Coniosporium uncinatum* (MC 557) as meristematic fungi.

2. Materials and Methods

2.1. Matrix preparation

This study was conducted with ordinary white Portland cement CEM I 52.5 R. The water/cement mass ratio is 0.55. Hardened cement paste samples are shaped as parallelepipeds (dimension 1 × 2.5 × 8.5 cm). Samples were demolded 24 hours after casting and stored for 28 days at 100% relative humidity, at room temperature. Accelerated weathering is performed with carbonation only for 48 hours, or carbonation (48 hours) followed by leaching operation (28 days) as described by Wiktor et al. (2006).

2.2. Fungal strains and media

A. alternata (MC342) and *Exophiala* sp. (MC843) were isolated from Bhubaneswar temple in India in 2006 (unpublished data) using the adhesive tape method (MAT) as described by Urzì and Albertano (2001). A little square of adhesive tape (about 5 mm^2) was streaked in DRBC agar (Oxoid) and incubated at 28°C until growth. *C. uncinatum* MC557 was isolated from Carrara marble statue located in the court-yard of the Messina Museum, Italy (De Leo et al., 1999). These isolated strains were kept in the collection of Department of Life Sciences in Messina, Italy.

A. alternata was cultivated in solid medium (Potato Dextrose Agar – PDA, Oxoid) for 5 days at 26°C. *Exophiala* sp. and *C. uncinatum* were cultivated in liquid medium (Malt Extract Broth - MEB Oxoid) for 5 days at 26°C.

Nutritive medium used for biodeterioration test is composed of 1x concentrated Yeast Nitrogen Broth (YNB) (Difco) plus the addition of Glucose 0.01%.

2.3. Fungal units suspension

Fungal colonies growing in solid medium were scraped from the agar surface and conidia suspended in physiological solution (0.9% of NaCl in distilled water), while the cultures in liquid medium were directly transferred in physiological solution. Each suspension was centrifuged 15 minutes at 2095g (3000 rpm with a Beckman and Coulter Allegra X-12R centrifuge – 4750 A rotor). Centrifugation pellet were put in suspension in 1 ml of physiological solution. The number of fungal units was determined through a direct microscopic count in a counting chamber (Bürker chamber, ProSciTech, Australia) and adjusted at a concentration of 8.7×10^5 fungal units/ml in YNB 1x + Glucose 0.01% medium.

2.4. Biodeterioration test

2.4.1. Experimental set up

Polyethylene boxes were used for test 9.5 x 9.5 x 9.5 cm. In order to keep humidity inside box, bottom was covered by vermiculite, paper sheet was disposed on it to avoid the direct contact between specimens and vermiculite (Fig. 1). Boxes were autoclaved 15 minutes at 120°C, then

sterile water was added on vermiculite to wet it. Two specimens of each matrix were disposed in each box.

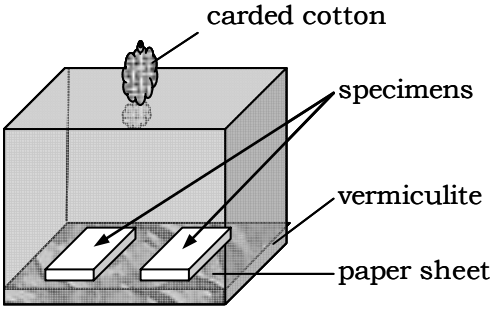

Figure 1 : Experimental set up for biodeterioration test

2.4.2. Inoculation

Each specimen was inoculated with 1.5 ml of fungal units suspension, except controls (only 1.5 ml of sterile medium). Inoculation was performed in duplicate, thus for each strain, 6 specimens were inoculated (2 unweathered, 2 carbonated, 2 carbonated and leached), and placed in 3 different boxes. Boxes were incubated at 26°C.

2.5. Analyses

2.5.1. Stereomicroscopy observations

In order to monitor fungal growth during test, the surface of each specimen was observed once a week with stereomicroscope (Leica, Wild M10).

2.5.2. Sampling of specimens

After four weeks of incubation, one specimen of each box was taken and broken in small pieces for Periodic Acid Schiff staining and Scanning Electron Microscopy (Fig 2).

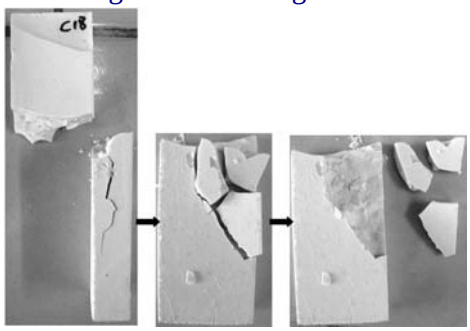

Figure 2 : Example of specimen broken to carry out PAS staining and SEM observations to assess the microbial colonisation

2.5.3. Periodic Acid Schiff (PAS) staining

PAS staining was performed on samples as reported by Urzì and Albertano (2001). Using this procedure, compounds such as extracellular polymeric substances (EPS), glycogen, starch, cellulose, chitin, mucin, protein-carbohydrates complexes, and glycolipids appear red. Samples were fixed with 70% (v/v) ethanol for 2 hours, then first transferred in 1% (w/v) periodic acid for 5-8 min then in ethanol 70% (v/v) for 5min. Samples are rinsed 5 min with distilled water and transferred 10 min to Schiff's reagent, then transferred to 0.6% (w/v) sodium metabisulfite 3 min 2 times. They were washed twice in distilled water for 5 min each, and then transferred to 70% (v/v) ethanol for 35 min.

2.5.4. Scanning Electron Microscopy (SEM)

Fixation of samples was performed overnight at 4°C in buffered aldehyde fixative 2% (w/v) formaldehyde (P6148, Sigma). Samples were then washed 3 times in 0.01M phosphate buffer (0.01M NaH2PO4.H2O, 0.01M Na2HPO4, pH 7.2) for 10 min each. Dehydration was made in graded ethanol series (70, 85, 95% (v/v) and in anhydrous ethanol (100%) for 15 minutes each). Samples were air dried and then coated with gold for SEM observation (JEOL 840 SEM).

3. Results and discussion

First, relating to results from stereomicroscopy observations, no microbial growth was observed on controls for all specimens (weathered or not). In the same way, no microbial development was noticed on the surface of non-weathered specimens, no matter the inoculated strain.

For carbonated specimens, the growth of *Exophiala* sp. was noticed in the third week of incubation. *A. alternata* development started in the first week of incubation, and the growth increases until the fourth week. Hyphae and spores production were observed on the specimens' surface (Fig. 3d,e). Development of *C. uncinatum* was noticed in the second week of incubation and increased until the fourth week. Development of a pink coloured contamination was observed on the surface of specimens inoculated with *Exophiala* sp., *A. alternata* and *C. uncinatum*. Nevertheless, the presence of this contamination doesn't seem to interfere with fungal development.

Relating to carbonated then leached specimens, the strains grew faster; and therefore the development of *Exophiala sp.*, *A. alternata* and *C. uncinatum* was observed from the first week of incubation. After four weeks of incubation, *Exophiala* sp. growth was characterized by numerous spots on surface (Fig. 3b); the one of *C. uncinatum* was homogeneous and increased from the first until the fourth week (Fig. 3c).

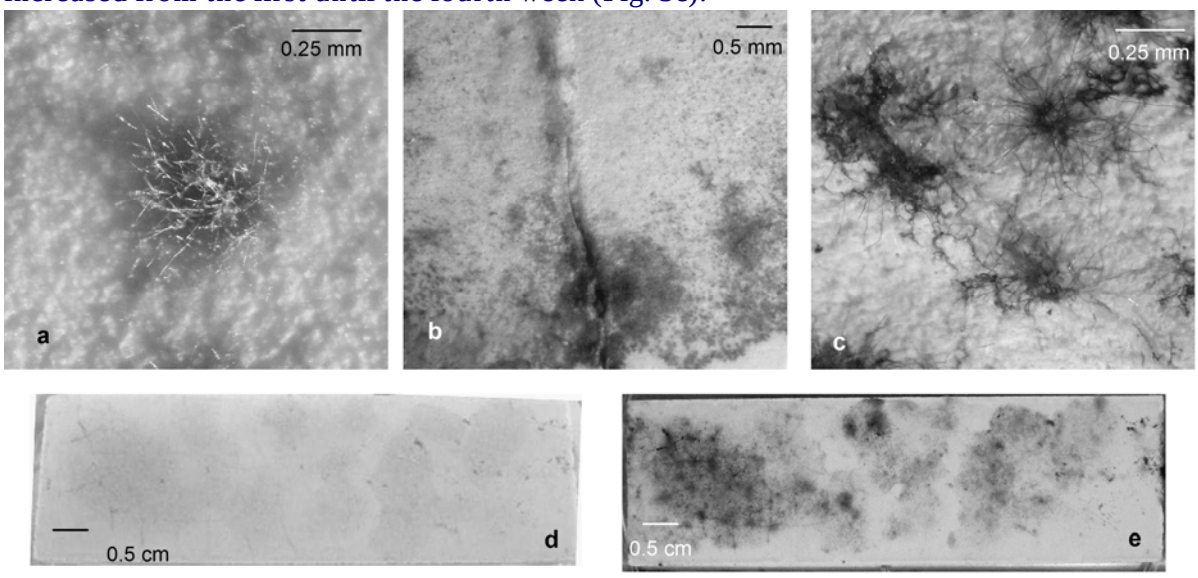

Figure 3 : Direct observations of specimens during biodeterioration test, a) and b) carbonated then leached specimen inoculated with Exophiala sp. after 1 week a), after 4 weeks b), c) carbonated then leached specimen inoculated with Coniosporium uncinatum after 4 weeks, d) and e) Carbonated specimen inoculated with Alternaria alternata after inoculation d), after 4 weeks (e).

Decrease of surface pH increases considerably matrix bioreceptivity, microbial colonisation is observed on some carbonated specimens and on all carbonated then leached specimens.

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Carbonation is the most common chemical reaction influencing cement-based materials in natural environmental scenarii (Macias et al., 1997; Gervais et al., 2004) that's why accelerated weathering of matrix is generally performed by carbonation (Dubosc, 2000, Shirakawa et al., 2003; de Moraes Pinheiros and Ribas Silva, 2003). But cement-based materials are also exposed to the elements (humidity, acidic rain, snow...) which leads to cement compounds leaching (Barbieri Albert, 2002). In our case, carbonation is followed by leaching operation. This allows us to obtain a surface pH about 8.8 after 30 days, and also to have two ways of weathering involved in natural weathering of materials (Wiktor, 2008).
Staining with periodic acid Schiff's reagent provides interesting results. All the controls (with and without the addition of medium, non-weathered, carbonated, carbonated then leached) appeared, after PAS staining, pale purple coloured which points out the absence of microbial growth. For carbonated specimens, a deep purple coloration is observed only for sample exposed to *A. alternata*. For carbonated then leached specimens, all samples are coloured in deep purple in homogeneous way and all over the exposed surface. This confirms previous observations, that leached specimens exhibited a higher microbial colonisation. PAS staining allows a better visualization of microbial development extent: in fact, PAS staining reveals that microbial activity is spread not only all over the surface but also, as demonstrated by observations of cross section (Fig. 4) that fungi penetrate within the matrix.

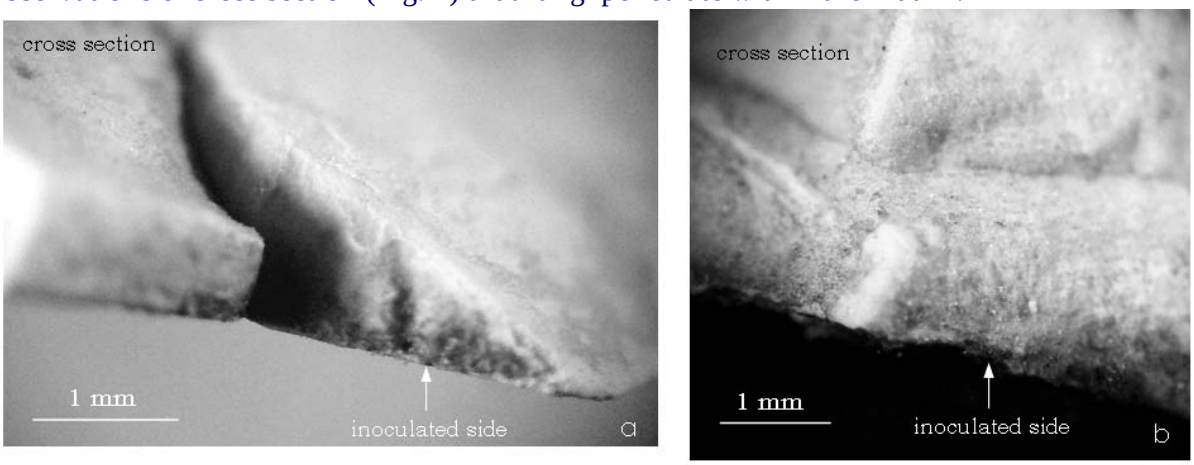

Figure 4 : Observations with stereomicroscope of samples after PAS staining, a) leached control, b) leached sample inoculated with Coniosporium uncinatum

SEM observations of the surface and the cross section were performed. The most interesting observations were noticed for carbonated then leached samples. In particularly, for samples inoculated with *A. alternata* and with *C. uncinatum*, hyphae were observed within the matrix, they penetrated through the cracks generated with accelerated weathering of the matrix (Fig. 5). From these observations depth of microbial colonisation can be estimated about 130 µm.

All the reference strains used where strains isolated from rocks and stone and thus already well adapted to survive on decayed stones. The strains presented different patterns of growth: *Exophiala* sp. develops from spots whereas *A. alternata* produces spores and also develops mycelium; the use of *C. uncinatum* was particularly encouraging as it presented a good growth all over the incubation time.

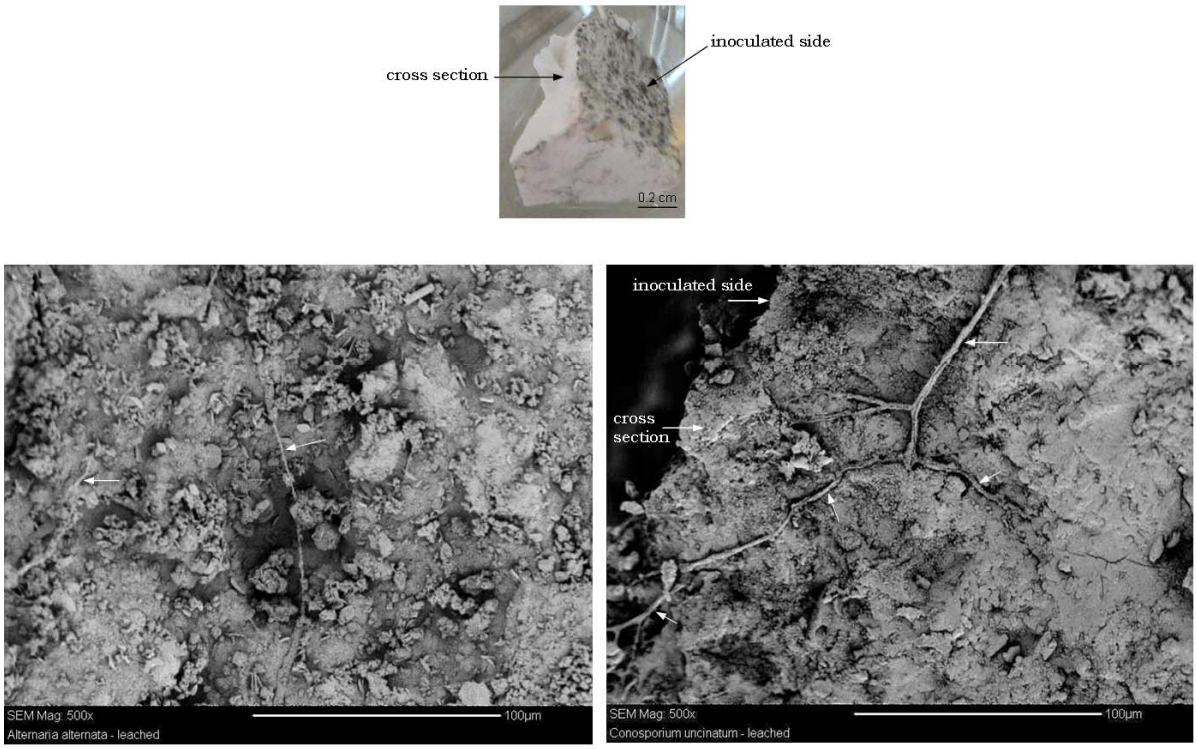

Figure 5 : SEM observations of cross sections of leached samples, arrows indicate hyphae localisation.

The accelerated laboratory test developed in this study permits to obtain a rapid fungal development on cement specimens. Three months of experiments only are needed to obtain first results, which is rather shorter than other test developed to date to study fungal biodeterioration. Results are mainly related to aesthetical biodeterioration.
This accelerated laboratory test may be used in different applications. Nowadays cement based materials contain more and more organic species used as adjuvant in order to enhance materials properties, but it brings organic matter to the matrix, and thus could enhance its primary bioreceptivity. Test can be used with short time of incubation, 4 weeks as described in this paper, to test and to compare bioreceptivity of cement based materials. On the other hand, accelerated test used with several months of incubation should allow to study mechanisms involved in biodeterioration, and also the impact of microbial growth on matrix properties, using microindentation for example.

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