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Fluorometric detection and estimation of fungal biomass on cultural heritage materials ☆

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ABSTRACT

A wide variety of cultural heritage materials are susceptible to fungal deterioration. The paper, canvas, and stone constituents of our cultural heritage are subjected to harmful physical and chemical processes as they are slowly consumed by fungi. Remediation of fungal contamination can be costly and risk further damage to cultural artifacts. Early detection of fungal growth would permit the use of relatively noninvasive treatments to remediate fungal contamination before visible or lasting damage to the object has occurred. Current methods used for the detection and measurement of microbial biomass, such as colony counts, microscopic biovolume estimation, and ergosterol analysis are expensive and time consuming, or are inappropriate for use with fungi. Beta-*N*-acetylhexosaminidase (3.2.1.52) activity provides a reliable estimation of fungal biomass in soil and on building materials. Adapted for use on cultural heritage materials' fluorogenic 4-methylumbelliferyl (MUF) labeled substrate *N*-acetyl-beta-D-glucosaminide (NAG) was used to detect beta-*N*-acetylhexosaminidase activity in the fungus *Aspergillus niger*. Fluorescence increased linearly with fungal biomass and the sensitivity of the assay was comparable to other biochemical techniques. The fluorometric assay was used to monitor fungal biomass on a variety of cultural heritage materials non-destructively, and without the introduction of chemicals or solvents to the surfaces.

1. Introduction

Fungi can colonize a variety of cultural heritage materials (Fig. 1). Canvas and paper, the cellulosic substrata of easel paintings and historic documents, can be stained and degraded by fungi. Even stone, the ostensibly permanent medium of monuments, sculptures, and building facades can be weathered by intrusive fungal growth. The treatments required to eliminate established fungal communities from these surfaces are costly and risk further damage to artifacts. Early detection of fungal growth would permit the use of relatively noninvasive treatments to remediate cultural artifacts before visible or lasting damage has occurred.

Paper is traditionally composed of cellulose, sizing agents, and fillers that create complex heterogeneous substrata conducive to fungal growth (Pinzari et al., 2006). Fungi can introduce blemishes that alter the aesthetic properties of paper (Meynell and Newsam, 1978; Florian and Manning, 2000; Pinzari et al., 2006), generate destructive acids (Sarantopoulou et al., 2006), and depolymerize the cellulose fibers that give paper its strength (Ponce-Jimenez et al., 2002; Piantanida et al., 2006; Zotti et al., 2008). The fungi *Aspergillus* spp., *Chaetomium* spp., *Alternaria* sp., and *Penicillium* spp. are all capable of cellulose degradation (Ponce-Jimenez et al., 2002) and are often associated with library materials (Zyska, 1997; Michaelson et al., 2006; Rakotonirainy et al., 2007; Zotti et al., 2008).

The canvas used to create easel paintings is customarily made of woven cotton or flax fabric coated with sizing agents, ground materials, paints, and varnishes (Young and Hibbard, 1999). Fungi decolorize pigments (Berovic, 2003), disintegrate paint layers, and degrade the binding agents of easel paintings (Ciferri, 1999) as they derive nutrients from the organic material in these substances (Gettens et al., 1941; Strzelczyk, 1981). Fungi also attack the underlying canvas fibers of paintings with cellulitic and proteolytic enzymes. Members of the genera *Aspergillus* and *Penicillium* have been implicated in canvas deterioration, while *Aureobasidia* are suspect in the degradation of pigments on painted surfaces (Seves et al., 1996).

The stone used to create monuments, statues, and building facades possess convoluted surfaces that present large areas for fungal adhesion (Gorbushina, 2007). The growth of black, melanin-encrusted fungi can alter the aesthetic properties of stone (Sterflinger, 2005) while physical deterioration occurs as fungal hyphae penetrate the surface, swell,

☆ This paper details a laboratory study conducted to examine the application of a fluorogenic assay for the early detection of fungal biomass on cultural heritage materials. We utilized fluorogenic 4-Methylumbelliferyl labeled substrate N-acetyl-Beta-D-glucosaminide to detect beta-*N*-acetylhexosaminidase activity in *Aspergillus niger*. We demonstrated that there was a linear relationship between *A. niger* biomass and fluorescence. Sensitivity of the assay was comparable to other biochemical techniques used to detect and quantify fungal biomass. The assay was applied to paper, canvas, and marble substrata and permitted rapid and reliable detection of fungal biomass. Use of this assay could prevent the aesthetic and physical damage done to cultural materials by fungal growth. The assay has the potential to assist conservators in quickly detecting fungal contamination and monitoring fungal growth on cultural heritage materials.

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deflate, and secrete acids (Sert et al., 2007). These events subject stone to physical and chemical stresses that hasten its disintegration. Chemoorganotrophic strains of *Exophiala*, *Penicillium*, *Aspergillus*, *Cladosporium*, *Alternaria*, *Aureobasidium*, *Ulocladium*, and *Phoma* that excrete organic acids or oxidize iron and manganese from minerals have been isolated from stone (Warscheid and Braams, 2000).

Fungal growth on a cultural artifact can lead to irreparable damage if not detected and treated. Detection is often delayed until altered aesthetics or odors, the first outward signs of fungal contamination, are noticed (Garg et al., 1995; Ciferri, 1999; Rakotonirainy et al., 2003). Current early fungal detection methods that involve direct microscopic observation, isolation on nutrient media, and genetic techniques can be employed, but are often incompatible with fragile works of art, uninformative, or require the expertise of trained molecular biologists. Quantitative methods that measure fungal ergosterol, phospholipid fatty acids, hyphal biovolume, and ATP activity can be time consuming, tedious, and possess a number of pitfalls that make them impractical for conservation scientists (Gessner and Newell, 2002). A fluorometric method based on the activity of beta-*N*-acetylhexosaminidase (3.2.1.52), an enzyme constitutively expressed in a diverse assortment of filamentous fungi, offers a more rapid means of fungal detection (Reeslev et al., 2003). Originally developed to measure fungal biomass in soil (Miller et al., 1998) fluorescent *N*-acetyl-beta-D-glucosamine could be adapted to detect and quantify fungal biomass on cultural heritage materials.

Fluorogenic 4-methylumbelliferyl-labeled *N*-acetyl-beta-D-glucosamine was used as a substrate to detect the beta-*N*-acetylhexosaminidase activity of the fungus *Aspergillus niger* on paper, canvas, and marble. Fungal biomass was non-destructively collected from the surface of these fragile substrata without the introduction of chemicals or solvents and assayed for beta-*N*-acetylhexosaminidase activity. The assay was able to detect minute (μg) quantities of *A. niger* biomass in less than 60 min and monitor the development of fungal biomass on paper, canvas, and marble over time.

2. Materials and methods

2.1. Standard curve development

A. niger was grown at 22 °C for 5-7 days in 500 ml volumes of Difco™ Tryptic Soy Broth (Becton-Dickinson and Co., Sparks, MD). The stationary phase *A. niger* culture was filtered through Whatman® cellulose nitrate filters (Whatman Lt., Maidstone, Kent, UK), rinsed, and resuspended in 50 mM Tris maleate (pH 5). The resuspended *A. niger* pellets were homogenized in a 12 speed Osterizer® homogenizer (Jarden Corp., Rye, NY) at high speed for 20 s. 0.001, 0.01, 0.02, 0.05, 0.075, 0.1, and 0.25 ml samples of the homogenized *A. niger* suspension that corresponded to 1.70, 17.03, 34.07, 85.16, 127.75, 170.33, and 425.83 μg respectively, of *A. niger* biomass were diluted to 2 ml with 50 mM Tris maleate (pH 5) and immediately assayed for beta-*N*-acetylhexosaminidase activity. Replicate 50 ml samples of the homogenized *A. niger* suspension were filtered though pre-weighed cellulose nitrate filters, dried at 65 °C for 48 h, and biomass dry weight determined. Three replicates were used for the calculation of *A. niger* biomass dry weight and measurement of beta-*N*-acetylhexosaminidase activity. A conversion factor of 0.379 μg/unit of fluorescence that could be used to estimate fungal biomass from the fluorescence data was calculated from linear regression analysis of the data.

2.2. Beta-N-acetylhexosaminidase assay

4-methylumbelliferyl-*N*-acetyl-beta-D-glucosaminide (MUF-NAG) (Sigma-Aldrich Co., St. Louis, MO) was used to detect beta-*N*-acetylhexosaminidase activity (Miller et al., 1998). All assays were conducted in 2 ml of 50 mM Tris maleate (pH 5). *A. niger* was added to the Tris maleate and briefly vortexed. Two-hundred microliters of MUF-NAG (200 μM) was added to the *A. niger* suspension, vortexed briefly, and incubated at 22 °C for 30 min. Ice-cold EtOH (2 ml) was added to quench the reaction. The pH was raised with the addition of 470 μl of 45 um Tris–HCl (pH 10). Samples were centrifuged at 10,000×*g* for 2 min to pellet any particulates. The centrifuged reaction mixture was divided into three, 300 μl aliquots and transferred to a 96-well plate. Fluorescence was measured with a SpectraMax® Plus^{384} plate reader (Molecular Devices, Sunnyvale, CA) at 377 nm excitation and 446 nm emission.

2.3. Inoculum preparation and experimental setup

Paper, canvas, and marble coupons inoculated with identical quantities of *A. niger* conidia were used to simulate contaminated cultural heritage materials and provide a standardized set of conditions that could be replicated, and used to generate data suitable for statistical analysis. *A. niger* was plated onto Difco™ Nutrient Agar (Becton-Dickinson and Co., Sparks, MD) and incubated at 22 °C for 5–7 days. Conidia were harvested in sterile 0.05% Tween® 80 (Sigma-Aldrich, St. Louis, MO) by pipetting a 250 μl volume of the solution onto the surface of the mycelium. The conidial suspension was briefly centrifuged and the resulting pellet resuspended in dilute Difco™ Tryptic Soy Broth (TSA) (Becton-Dickinson and Co., Sparks, MD) to a concentration of 2×10^4 or 2×10^5 conidia/ml. Aliquots (20 μl) of the 2×10^4 conidia/ml suspension were applied to sterile 23 cm^2 coupons of aged book paper or canvas while 2 μl aliquots of the 2×10^5 conidia suspension were applied to the surface of EtOH sterilized marble blocks (2l×2w×0.75h cm). The paper and canvas coupons were dried under laminar flow, aseptically transferred to TSA plates which were sealed with Parafilm® (Pechiney Plastic Packaging, Menasha, WI), and incubated at 30 °C. The marble blocks were dried under laminar flow, inverted, and aseptically transferred to TSA media (Fig. 2). Prior to transfer of the marble coupons a cavity was introduced into the agar surface with a sterile 1.5 cm cork borer. Transfer of the inverted marble coupon to a position atop the cavity in the TSA maintained humid, nutrient rich conditions on the marble surface without permitting direct contact between the conidia and the TSA. Samples were incubated for 12, 18, 24, or 30 h intervals. Each sample was assayed for beta-*N*-acetylhexosaminidase activity immediately after incubation.

2.4. Beta-N-acetylhexosaminidase assay on paper, canvas, and marble

Polyurethane swabs of the BBL™ CultureSwab™ EZ Collection and Transport System (Becton-Dickinson and Co., Sparks, MD) were used to remove *A. niger* from a 1 cm^2 area of each paper, canvas, and marble coupon without damage to, or removal of the substratum. A 1 cm^2 stencil cut from a polystyrene weigh boat was utilized to ensure uniform sample collection from multiple coupons. The stencil was sanitized prior to the collection of each sample with a PDI® alcohol prep pad (Professional Disposables Int., Orangeburg, NY). Swabs with adhered *A. niger* biomass and negative control swabs were returned to the round-bottom transport tubes of the BBL™ CultureSwab™ EZ Collection and Transport System. The transport tubes were subsequently filled with 2 ml of 50 mM Tris maleate (pH 5) and vigorously mixed. After the addition of 200 μl MUF-NAG each reaction mixture was briefly vortexed and incubated at 22 °C for 30 min. Reactions were quenched with 2 ml of ice-cold EtOH followed by a brief vortex. The pH of the quenched reactions was adjusted with 470 μl of 45 um Tris–HCl (pH 10) followed by a final brief vortex. A 1 ml aliquot of each reaction was transferred to a 1.5 ml centrifuge tube and centrifuged at 10,000×*g* for 2 min. The centrifuged reaction mixtures were each divided into three, 300 μl replicates and transferred to a 96-well plate. Fluorescence of each replicate was measured with the SpectraMax® Plus^{384} plate reader at 377 nm excitation and 446 nm emission.

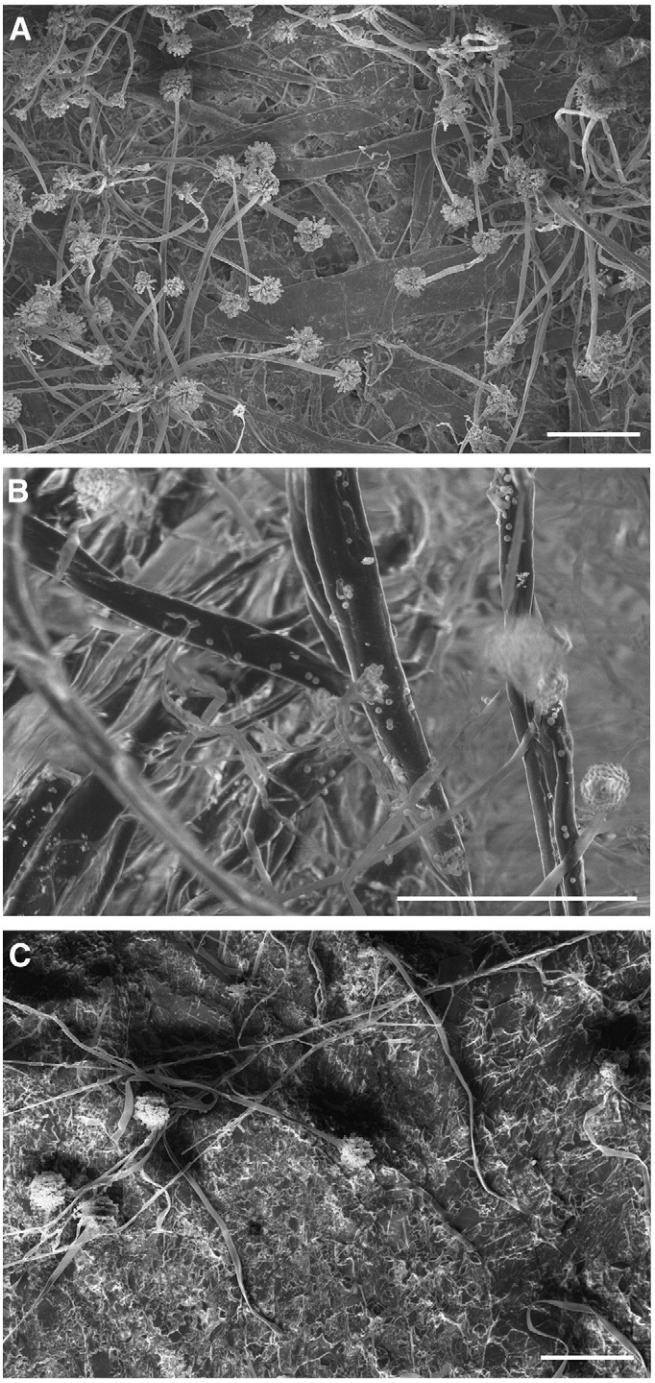

Fig. 1. Scanning Electron Micrographs of 48 hour *A. niger* cultures grown on book paper (A), canvas (B), and Carrera marble (C). The scale bar represents 100 µm.

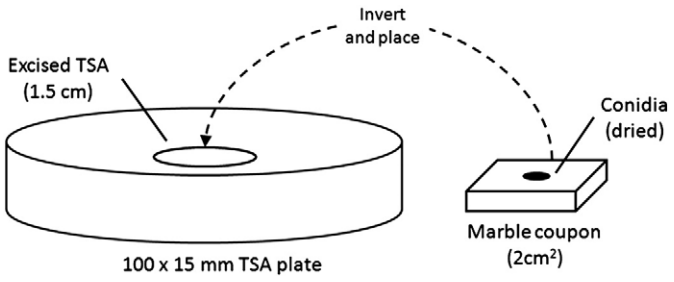

Fig. 2. Schematic representation of *A. niger* incubation on marble coupons. A 1.5 cm diameter core of TSA was removed from the plate with a sterile cork borer. A marble coupon previously inoculated with *A. niger* conidia was inverted and placed atop the cavity.

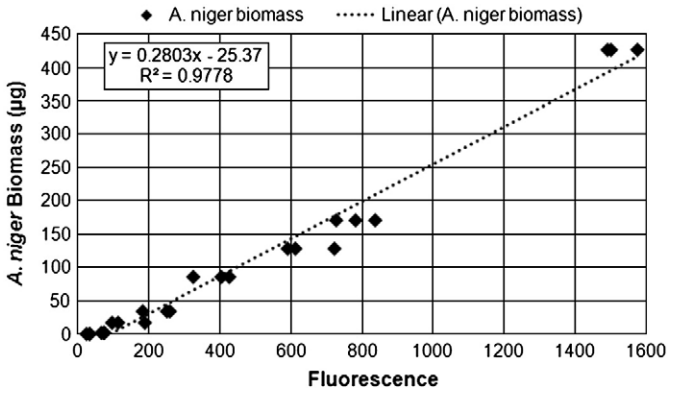

Fig. 3. The relationship between *A. niger* biomass and fluorescence. Data points represent 0, 1.70, 17.03, 34.07, 85.16, 127.75, 170.33, and 425.83 µg of *A. niger* biomass ($R^2 = 0.9778$, $p < 0.001$).

3. Results

3.1. Fungal biomass and fluorescence

Fungal biomass was related to fluorescence output through the construction of a calibration curve (Fig. 3). A consistent linear relationship ($R^2 = 0.9778$) between beta-*N*-acetylhexosaminidase activity and fluorescence was maintained as the quantity of dry biomass increased from 1.70 to 425.83 µg. A conversion factor (0.379 µg of biomass/unit of fluorescence) derived from the slope of the linear regression permitted the estimation of *A. niger* biomass from the fluorescence data in later experiments.

3.2. Lower detection limit

The fluorescence generated from the beta-*N*-acetylhexosaminidase cleavage of the MUF-NAG substrate by progressively smaller quantities of *A. niger* biomass was used to resolve the lower detection limit of the assay (Fig. 3). The mean fluorescence generated by 1.7 µg of fungal biomass was significantly higher ($p < 0.05$) than the mean background fluorescence according to an analysis of variance (ANOVA) and established this value as the lower detection limit of the assay. Statistical analysis using ANOVA demonstrated that the mean fluorescence produced by the 1.7 µg and 17.03 µg replicates

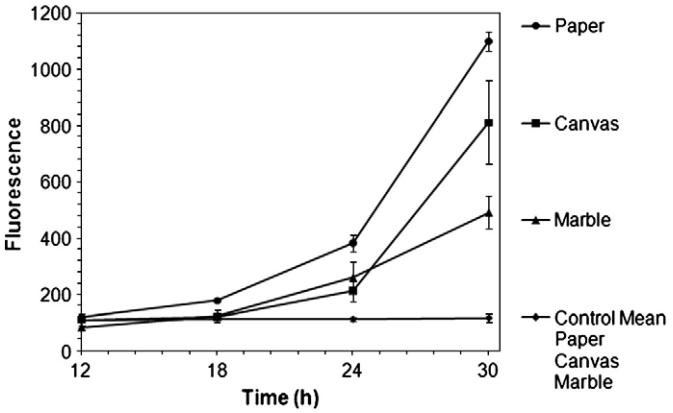

Fig. 4. Fluorescence generated from the beta-*N*-acetylhexosaminidase cleavage of MUF-NAG on paper, canvas, or marble coupons assayed after 12, 18, 24, and 30 h of incubation at 30 °C. Data points represent the means of three replicates. Standard deviations are indicated. The control mean data points represent the average of all the negative control reactions for each substratum at each time point ($n = 9$).

were not statistically different ($p = 0.09$). The mean fluorescence produced by the 17.03 µg replicates could be distinguished from the 34.07 µg replicates and all subsequent data points (Fig. 3). The resolution limit of the assay was 17.04 µg; the difference between 34.07and 17.03 µg of fungal biomass.

3.3. Application to cultural heritage materials

The utility of the beta-*N*-acetylhexosaminidase assay and its application to cultural heritage materials was tested with paper, canvas, and marble coupons inoculated with equal numbers of *A. niger* conidia (Fig. 4). Fungal biomass was detectable on paper after 18 h of incubation at 30 °C on tryptic soy agar. Twenty-four hours of incubation were required before fungal biomass could be detected on canvas and marble. The differential kinetics of *A. niger* development on paper, canvas, and marble between 12 and 30 h of incubation were evident. The fluorescence generated from the beta-*N*-acetylhexosaminidase cleavage of the MUF-NAG substrate increased most rapidly (slope = 10.46) and to the greatest magnitude (1098.84 fluorescence units) on paper. The increase in fluorescence on canvas ($m = 7.29$) and marble ($m = 4.52$) lagged. After 30 h of incubation mean fluorescent values of 810.24 and 490.14 were recorded on the canvas and marble coupons, respectively, and did not achieve the levels observed on paper. After a relatively sharp increase in fluorescence between 12 and 24 h, the increase on marble appeared to slow (Fig. 4).

3.4. Variations introduced by the canvas substratum

Negative control reactions conducted on marble and paper generated fluorescence values between 106.29 and 117.23 respectively. Negative control reactions conducted on canvas uniformly generated fluorescence levels between 375.29 and 429.59. The mean difference between these negative control groups corresponded to 289 units of fluorescence and was deemed inherent to the canvas substratum upon failure to culture organisms from the negative control coupons. All fluorescence data recorded from the assays performed on the canvas coupons were normalized to the data collected from the paper and marble coupons. This permitted direct comparison between all three substrata (Fig. 4).

4. Discussion

Fluorogenic 4-Methylumbelliferyl-labeled *N*-acetyl-beta-D-glucosamine was used as a substrate to rapidly detect minute quantities of fungal biomass in liquid culture and on paper, canvas, and marble. In liquid culture the amount of beta-*N*-acetylhexosaminidase activity and the dry biomass of stationary phase *A. niger* displayed a linear relationship (Fig. 3). Previous studies have found that beta-*N*-acetylhexosaminidase was constitutively expressed in fungi and accurately represents fungal biomass in both the growth and the stationary phase (Rast et al., 1991; Cirano and Peberdy, 1993). Beta-*N*-acetylhexosaminidase activity was also significantly correlated with other fungal detection methods including measurements of phospholipid, ergosterol, and laccase (Matcham et al., 1985; Reeslev et al., 2003)

The beta-*N*-acetylhexosaminidase assay detected the fluorescence produced by 1.7 µg of stationary phase *A. niger* biomass in liquid culture and directly detected *A. niger* biomass on paper, canvas, and marble substrata after 18–24 h of growth (Fig. 4). Fungal beta-*N*-acetylhexosaminidase activity could be assayed in less than 60 min, and required relatively basic laboratory skills and equipment. *A. niger* biomass could be detected on book paper, canvas, and marble before it was visible to the unaided human eye with a sensitivity that equaled or exceeded levels attained on paper with ATP assays, or measurements of ergosterol content (Fabbri et al., 1997; Rakotonirainy et al., 2003). PCR amplification of fungal DNA and fluorescence *in situ* hybridization have been used to detect fungi on culturally significant marble (Urzi et al., 2003; Capitelli et al., 2007), but these methods, along with culture-based methods and microscopic hyphal biovolume measurements, require sophisticated equipment, trained personnel, and sufficient time to collect data and interpret results.

The use of fluorogenic 4-methylumbelliferyl-labeled substrates has been documented on stone (Hirsch et al., 1995), but the method required the direct application of buffers and substrates to the surface. The use of BBL™ CultureSwab™ EZ Collection and Transport System swabs permitted the acquisition of fungal biomass from sensitive cultural materials without the removal of any sensitive underlying materials or the introduction of chemicals or solvents to the surface. The portable round-bottom transport tubes provided the flexibility to monitor remote or immobile artifacts. Conservators could initiate assays on-site and the reactions allowed to proceed during transport to a suitable laboratory. Reactions could also be terminated with EtOH and transferred to more distant facilities. Fluorescence could also be measured on-site with a portable fluorometer.

Proper negative controls and knowledge of the substrata are essential for accurate estimation of fungal biomass. Certain substrata may contain constituents that autofluoresce and interfere with data collection. The canvas used in this study was coated with a gesso of unknown composition, though modern gesso is commonly composed of calcium carbonate and a pigment suspended in an acrylic latex matrix. Latex is known to autofluoresce and has been employed in a variety of clinical fluorescent microscopy studies. Thus, utilization of proper negative controls will be needed to avoid the false positive signals that may arise from sample autofluorescence.

The ability to estimate fungal biomass on cultural heritage materials would provide conservators a tool to conduct routine monitoring of at-risk materials in their collections, and evaluate the effectiveness of remediation efforts. Practitioners should remember that the results obtained from fluorescence data converted into biomass measurements can only be considered estimates because conversion factors derived from the beta-*N*-acetylhexosaminidase activity established under laboratory conditions are not identical to those on a cultural artifact. The effects of bacterial contamination, the presence of dust, dirt or grime, and fluctuations in environmental conditions on beta-*N*-acetylhexosaminidase expression in a complex fungal community remain unknown. However, beta-*N*-acetylhexosaminidase activity in bacteria is limited to a nutritional role and is restricted to a limited number of species (Gooday, 1990). Experimental evidence based on ergosterol concentrations suggest that the data derived from fungi grown under artificial conditions frequently does not differ significantly from fungi grown under natural conditions (Newell, 1994) and are still valuable (Gessner and Newell, 2002).

The basic requirement of a rapid, non-destructive, easy to use test with sufficient sensitivity to detect limited (µg) quantities of fungal biomass on cultural artifacts was within the capabilities of this assay. The assay was capable of rapid (<60 min) detection and estimation of fungal biomass on a variety of cultural heritage materials in the laboratory and has the potential to assist conservators in the assessment of fugal remediation techniques. Future work conducted in association with conservators and archivists will apply this method to cultural heritage artifacts in libraries, archives, and museums.

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References

Berovic, M., 2003. Biodeterioration studies on pastels and oil-based paintings. In: Koestler, R.J., Koestler, V.H., Charola, A.E., Nieto-Fernandez, F.E. (Eds.), Art, Biology, and Conservation: Biodeterioration of Works of Art. Metropolitan Museum of Art, New York, USA, pp. 50–59.

Capitelli, F., Principi, P., Pedrazzani, R., Toniolo, L., Sorlini, C., 2007. Bacterial and fungal deterioration of the Milan Cathedral marble treated with protective synthetic resins. Sci. Total Environ. 385, 172–181.

Ciferri, O., 1999. Microbial degradation of paintings. Appl. Environ. Microbiol. 65, 879–885.

Cirano, J.U., Peberdy, J.F., 1993. Effect of carbon source on chitobiase production by *Trichoderma harzianum*. Mycol. Res. 97, 45–48.

Fabbri, A.A., Ricelli, A., Brasini, S., Fanelli, C., 1997. Effect of different antifungals on the control of paper biodeterrioration caused by fungi. Int. Biodeterior. Biodegrad. 39, 61–65.

Florian, M.-L.E., Manning, L., 2000. SEM analysis of irregular fungal fox spots in an 1854 book: population dynamics and species identification. Int. Biodeterior. Biodegrad. 46, 205–220.

Garg, K.L., Jain, K.K., Mishra, A.K., 1995. Role of fungi in the deterioration of wall paintings. Sci. Total Environ. 167, 255–271.

Gessner, M.O., Newell, S.Y., 2002. Biomass, growth rate, and production of filamentous fungi in plant litter, In: Hurst, C.J., Crawford, R.L., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D. (Eds.), Manual of Environmental Microbiology, 2nd. ASM Press, Washington D.C., USA, pp. 390–408.

Gettens, R.J., Pease, M., Stout, G.I., 1941. The problem of mold growth in paintings. Tech. Stud. Fine Arts 9, 127–143.

Gooday, G.W., 1990. The ecology of chitin degradation. In: Marshall, K.C. (Ed.), Advances in Microbial Ecology, vol. 11. Plenum Press, New York, N.Y, pp. 387–430.

Gorbushina, A., 2007. Life on the rocks. Environ. Microbiol. 9, 1613–1631.

Hirsch, P., Eckhardt, F.E.W., Palmer Jr., R.J., 1995. Methods for the study of rock-inhabiting microorganisms—a mini review. J. Microbiol. Methods 23, 143–167.

Matcham, S.E., Jordan, B.R., Wood, D.A., 1985. Estimation of fungal biomass in a solid substrate by three independent methods. Appl. Microbiol. Biotechnol. 21, 108–112.

Meynell, G.G., Newsam, R.J., 1978. Foxing, a fungal infection of paper. Nature 274, 466–468.

Michaelson, A., Pinzari, F., Ripka, K., Lubitz, W., Pinar, G., 2006. Application of molecular techniques for identification of fungal communities colonizing paper material. Int. Biodeterior. Biodegrad. 58, 133–141.

Miller, M., Palojarvi, A., Rangger, A., Reeslev, M., Kjoller, A., 1998. The use of fluorogenic substrates to measure fungal presence and activity in soil. Appl. Environ. Microbiol. 64, 613–617.

Newell, S.Y., 1994. Total and free ergosterol in mycelia of saltmarsh ascomycetes with access to whole leaves or aqueous extracts of leaves. Appl. Environ. Microbiol. 60, 3479–3482.

Piantanida, G., Pinzari, F., Montanari, M., Bicchieri, M., Coluzza, C., 2006. Atomic force microscopy applied to the study of Whatman paper surface deteriorated by a cellulolytic filamentous fungus. Macromol. Symp. 238, 92–97.

Pinzari, F., Pasquariello, G., De Mico, A., 2006. Biodeterioration of paper: a SEM study of fungal spoilage reproduced under controlled conditions. Macromol. Symp. 238, 57–66.

Ponce-Jimenez, M., Lopez-Dellamary Toral, F.A., Fornue, E.D., 2002. Antifungal protection and sizing of paper with chitosan salts and cellulose ethers. J. Am. Inst. Conserv. 41, 243–254.

Rakotonirainy, M.S., Heraud, C., Lavédrine, B., 2003. Detection of viable fungal spores contaminant on documents and rapid control of the effectiveness of an ethylene oxide disinfection using ATP assay. Luminescence 18, 113–121.

Rakotonirainy, M.S., Heude, E., Lavédrine, B., 2007. Isolation and attempts of biomolecular characterization of fungal strains associated to foxing on a 19th century book. J. Cult. Herit. 8, 126–133.

Rast, D.M., Horsch, M., Furter, R., Gooday, G.W., 1991. A complex chitinolytic system in exponentially growing mycelium of *Mucor rouxii*: properties and function. J. Gen. Microbiol. 137, 2797–2810.

Reeslev, M., Miller, M., Nielson, K.F., 2003. Quantifying mold biomass on gypsum board: comparison of ergosterol and Beta-N-acetylhexosaminidase as mold biomass parameters. Appl. Environ. Microbiol. 69, 3996–3998.

Sarantopoulou, E., Kollia, Z., Gomoiu, I., 2006. Preventing biological activity of *Ulocladium* sp spores in artifacts using 157-nm laser. Appl. Phys. A. 83, 663–668.

Sert, H., Sumbul, H., Sterflinger, K., 2007. Microcolonial fungi from antique marbles in Perge/Side/Termessos (Antalya/Turkey). Antonie Van Leeuwenhoek 91, 217–227.

Seves, A.M., Sora, S., Ciferri, O., 1996. The microbial colonization of oil paintings. A laboratory investigation. Int. Biodeterior. Biodegrad. 37, 215–224.

Sterflinger, K., 2005. Black yeasts and meristematic fungi: ecology, diversity and identification. In: Rosa, C., Gabor, P. (Eds.), Yeast Handbook: Biodiversity and Ecophysiology of Yeasts. Springer, New York, USA, pp. 505–518.

Strzelczyk, A.B., 1981. Paintings and sculptures. In: Rose, A.H. (Ed.), Microbial Deterioration. Academic Press, London, pp. 203–234.

Urzi, C., De Leo, F., Donato, P., La Cono, V., 2003. Study of microbial communities colonizing hypogean monument surfaces using nondestructive and destructive sampling methods. In: Koestler, R.J., Koestler, V.H., Charola, A.E., Nieto-Fernandez, F.E. (Eds.), Art, biology, and conservation: biodeterioration of works of art, Metropolitan Museum of Art, pp. 316–325.

Warscheid, T., Braams, J., 2000. Biodeterioration of stone: a review. Int. Biodeter. Biodegr. 46, 343–368.

Young, C.R.T., Hibbard, R.D., 1999. Biaxial tensile testing of paintings on canvas. Stud. Conserv. 44, 129–141.

Zotti, M., Ferroni, A., Calvini, P., 2008. Microfungal biodeterioration of historic paper: preliminary FTIR and microbiological analyses. Int. Biodeterior. Biodegrad. 62, 186–194.

Zyska, B., 1997. Fungi isolated from library materials: a review of the literature. Int. Biodeterior. Biodegrad. 40, 43–51.