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Colorimetric micro-assay for accelerated screening of mould inhibitors☆

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ABSTRACT

Since current standard laboratory methods are time-consuming macro-assays that rely on subjective visual ratings of mould growth, rapid and quantitative laboratory methods are needed to screen potential mould inhibitors for use in and on cellulose-based products. A colorimetric micro-assay has been developed that uses XTT tetrazolium salt to enzymatically assess metabolic activity in mould spores, saving significant time and resources (i.e. wood specimens). Minimal inhibitory concentration (MIC_{90}) of isothiazolinone, a known mould inhibitor, in the XTT assay was the same or within a two-fold dilution of the MIC_{90} for two methods currently used to determine mould resistance of cellulose-based products. An ATP assay corroborated XTT assay findings; isothiazolinone appeared to be fungicidal rather than fungistatic to the spores of fungi used in this study. After 24 h exposure to the chemical, spores remained inactive indefinitely. The XTT assay would be a useful tool for screening mould inhibitors for numerous applications in addition to those of the forest products industry.

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1. Introduction

Under suitable environmental conditions, spores of mould fungi may germinate within 24–48 h. Cellulose-based products are an adequate food source for mould so wood, paper and engineered composites can be plagued with mould growth if they are chronically exposed to moisture and warm temperatures. Wood preservatives are not typically developed with mould inhibition in mind; rather, they are intended to inhibit destruction of wood products by wood decay fungi. But mould fungi often grow uninhibited and occasionally grow preferentially on wood that has been pressure-treated with wood preservatives, so research on mould inhibition has become increasingly important to the wood preservation industry. Two standard laboratory test methods to screen efficacy of mould inhibitors for wood products (American Society for Testing Materials , 2010; American Wood Protection Association Standards, 2010) require four to eight weeks for test results depending on the test method selected. The two standard methods most frequently used rely on subjective visual ratings of wood specimens. Accelerated quantitative methods to measure the efficacy of mould inhibitors are needed.

Colorimetric assays using tetrazolium were originally developed to verify vegetative viability of clinically important yeasts, such as *Candida* spp. and *Saccharomyces cervisiae*, and human pathogenic moulds, such as *Aspergillus fumigatus* (Levitz and Diamond, 1985; Kuhn et al., 2003). These assays utilized yellow tetrazolium salts, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) or 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT). Both tetrazolium salts are cleaved by mitochondrial dehydrogenases to form a formazan derivative. In the presence of an electron-coupling agent, the formazan derivative absorbs light in a specific nanometer range that can be read in a microplate reader (Altman, 1976; Hawser et al., 1998; Meletiadis et al., 2001). Colorimetric tetrazolium assays are reported to produce clear-cut endpoints for minimum inhibitory concentration (MIC) determination of antifungal agents (Hawser et al., 1998; Meletiadis et al., 2001).

A number of assay variations have been reported for XTT antifungal susceptibility of both yeasts and biofilms. Hawser et al. (1998) conducted susceptibility testing against numerous clinical isolates of *Candida* spp. using XTT and compared results with those from a standardized clinical test (National Committee for Clinical Laboratory Standards, 1997). Their results showed that one hundred percent of the isolates assayed by the two methods demonstrated agreement within two (2-fold) dilutions of the MIC obtained for amphotericin B, itraconazole, ketoconazole, fluconazole and flucytosine.

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Pierce et al. (2008) developed a method based on fungal biofilm development. In their model, biofilms of medically-important moulds and yeasts grown in a 96-well microtiter plate were subjected to antifungal drugs. The tetrazolium salt was added to measure the metabolic activities of cells within the biofilm. By this method, the rate of biofilm formation was shown to be reproducible and there were no statistical differences in replicate values of the susceptibility tests.

Clausen and Yang (2011) optimized parameters for the XTT microassay and demonstrated that the variability between fungal species can be used to demonstrate the relative resistance of different fungal species to several antifungal agents. Since isothiazolinone is commonly used by the wood preservation industry to inhibit mould growth on preservative treated wood, it was selected as the model compound for this study to compare laboratory mould resistance test methods.

The objectives of this study were 1) to compare the MIC_{90} for isothiazolinone using the XTT assay with the MIC_{90} for two standardized laboratory methods, 2) to verify XTT results with ATP production, and 3) to assess fungicidal versus fungistatic activity of isothiazolinone against mould spores using the XTT assay.

2. Materials and methods

2.1. XTT assay reagents

Growth medium supporting spore germination was RPMI 1640 medium (Moore et al., 1967) without sodium bicarbonate supplemented with L-glutamine and buffered with 165 mM morpholinepropanesulfonic (MOPS) acid, pH 7.0 (Sigma–Aldrich, St. Louis, MO) (Pierce et al., 2008). The tetrazolium salt, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2*H*-tetrazolium hydroxide (hereafter called XTT) (Sigma–Aldrich) was diluted in phosphate buffered saline, (PBS; 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4) (Sigma–Aldrich) to a concentration of 0.5 g l^{-1}, filter-sterilized and stored in 10 ml aliquots protected from light at −70 °C. A 10 mM stock of menadione (2-methyl-1,4 naphthoquinone) (Sigma–Aldrich) was prepared in 100% acetone and stored in 50 μl aliquots at −70 °C. Isothiazolinone (SUPATIMBER® H.E. 14, supplied by Viance, Charlotte, NC) served as the model mould inhibitor. Two-fold dilutions of isothiazolinone were prepared resulting in concentrations ranging 0–800 ppm. Isothiazolinone was diluted in RPMI for the XTT assay and in water for the American Wood Protection Association Standards (AWPA) and American Society for Testing Materials (ASTM) standardized test methods.

2.2. Spore inoculum

Mould fungi, *Aspergillus niger* 2.242 (provided by the University of Virginia School of Medicine, Charlottesville, Virginia, USA), *Penicillium chrysogenum* PH02 (provided by the USDA Forest Products Laboratory, Madison, Wisconsin, USA) and *Trichoderma atroviride* ATCC 20476 were maintained on 2% malt extract agar (Difco Laboratories, Detroit, Michigan, USA) and stored at 5 °C. Individual isolates were grown on 2% malt extract agar in Petri dishes and incubated at 27 °C for two weeks. Two different spore suspensions were prepared as described below:

For the XTT assay, spores from individual test fungi were collected by flooding the surface of a two-week-old culture with 10 ml of sterile PBS plus 0.025% Tween-20. The resulting spore suspension was removed via aspiration to a sterile container, washed twice in sterile PBS and collected by centrifugation at 3000 × *g* for 15 min each at 4 °C. Spores suspended in RPMI 1640 were counted with a hemocytometer and further diluted in RPMI to yield 1×10^6 spores ml^{-1}. In a preliminary study, 1×10^6 spores ml^{-1} of each test fungus was determined to be the optimal inoculum in the XTT assay (Clausen and Yang, 2011).

For both ASTM and AWPA standardized tests, spores from individual test fungi were collected in 10 ml of sterile deionized water (DI) according to ASTM standard D4445-10 (American Wood Protection Association Standards, 2010). Spores were counted and equal numbers of spores for each test organism were transferred to a spray bottle. The spore mixture was diluted with DI water to yield approximately 3×10^7 spores ml^{-1}. The spray bottle was adjusted to deliver 1 ml inoculum per spray and was shaken frequently during inoculation to ensure a homogeneous inoculum.

2.3. Wood specimen preparation

Southern pine sapwood specimens either 7 × 20 mm cross section × 7 cm long or 75 × 100 mm × 12.5 mm thick were cut from kiln-dried lumber and conditioned at 27 °C and 70% relative humidity (RH). Typically, mould testing involves dip-treating unseasoned wood, but to mimic methods for using isothiazolinone in wood preservatives, vacuum treatment of kiln-dried wood was used in this study. Ten random replicates of each size specimen were vacuum-treated for 40 min at −172 kPa submerged in each concentration of isothiazolinone test solution. Treated specimens were air-dried for 1 week prior to conditioning at 27 °C and 70% RH for 21 days.

2.4. XTT assay

For each mould isolate, 1×10^6 spores were pre-incubated in RPMI at 27 °C for 24 h. In a sterile flat-bottom polystyrene microplate, 80 μl of RPMI and 80 μl of saline were added to each well. Eighty μl of 2-fold dilutions of isothiazolinone ranging from (0–800 ppm) were prepared in sterile RPMI and added to each well ($n = 10$) with appropriate positive and negative control wells. The plate was sealed and incubated for 24 h at 37 °C. Following exposure to isothiazolinone, 80 μl XTT-menadione reagent was added to all wells of the plate which was then wrapped in foil and incubated for 3 h at 37 °C. Absorbance was read with a PowerWave XS 2 microplate reader (BioTek Instruments, Inc., Winooski, VT) at 490 nm.

2.5. ASTM D4445-10

Treated wood specimens (7 × 20 mm × 7 cm) ($n = 10$) were placed on top of a polyethylene mesh spacer in Petri dishes (150 × 25 mm) (B-D Falcon, Los Angeles, CA) containing 4 layers of blotting paper that was saturated with 30 ml DI water. Specimens were sprayed with 1 ml of mixed mould spore inocula, sealed in polyethylene bags to retain 100% RH, and incubated at 27 °C and 70% RH. Individual specimens were visually evaluated for mould growth after 4, 8, and 12 weeks of incubation on a scale of 0–5 with 0 indicating no mould growth and 5 indicating heavy mould growth on all sides of the specimen.

2.6. AWPA E24

Environmental chambers fabricated according to standard method AWPA E24 specifications were used to suspend treated wood specimens (75 × 100 mm × 12.5 mm) ($n = 10$) over non-sterile soil pre-inoculated with spores from the three test fungi. The test chambers were placed in a conditioning room at 27 °C. Treated specimens were vertically suspended across the width of the chamber over inoculated soil and sprayed with spores of the test fungi. Individual specimens were rated for mould growth on