International Biodeterioration & Biodegradation 77 (2013) 68–71

Contents lists available at SciVerse ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Colorimetric micro-assay for accelerated screening of mould inhibitors☆

Carol A. Clausen*, Vina W. Yang

USDA Forest Service, Forest Products Laboratory, One Gifford Pinchot Drive, Madison, WI 53726, USA

ARTICLE INFO

Article history:
Received 21 September 2012
Received in revised form
1 November 2012
Accepted 4 November 2012
Available online

Keywords:
XTT tetrazolium salt
Mould inhibitors
Micro-assay
Mould fungi
Spore germination

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.

Published by Elsevier Ltd.

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.

☆ The Forest Products Laboratory is maintained in cooperation with the University of Wisconsin. This article was written and prepared by U.S. Government employees on official time, and it is therefore in the public domain and not subject to copyright. The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

* Corresponding author. Tel.: +1 608 231 9253; fax: +1 608 231 9592.
E-mail addresses: clausen@wisc.edu, cclausen@fs.fed.us (C.A. Clausen), vyang@fs.fed.us (V.W. Yang).

0964-8305/$ – see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.ibiod.2012.11.005

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

a scale of 0–5 after 4, 8, and 12 wks incubation with 0 indicating no mould growth and 5 indicating heavy mould growth on all sides of the specimen.

2.7. ATP assay

An ATP assay was conducted to confirm that complete inhibition of metabolic activity occurred in the mould spores following exposure to isothiazolinone. Adenosine triphosphate (ATP) was determined using the ATP Lite luminescence detection assay kit (Perkin Elmer Life Sciences, Boston, MA). Spores (1×10^6 ml^{-1}) from individual test fungi were incubated with the minimum inhibitory concentration (MIC_{90}) of isothiazolinone (50 ppm) for 24 h at 37 °C. Following incubation, spores were pelleted by centrifugation (2500 × *g*), washed twice with RPMI to remove isothiazolinone and assayed for ATP production according to the ATP Lite assay protocol.

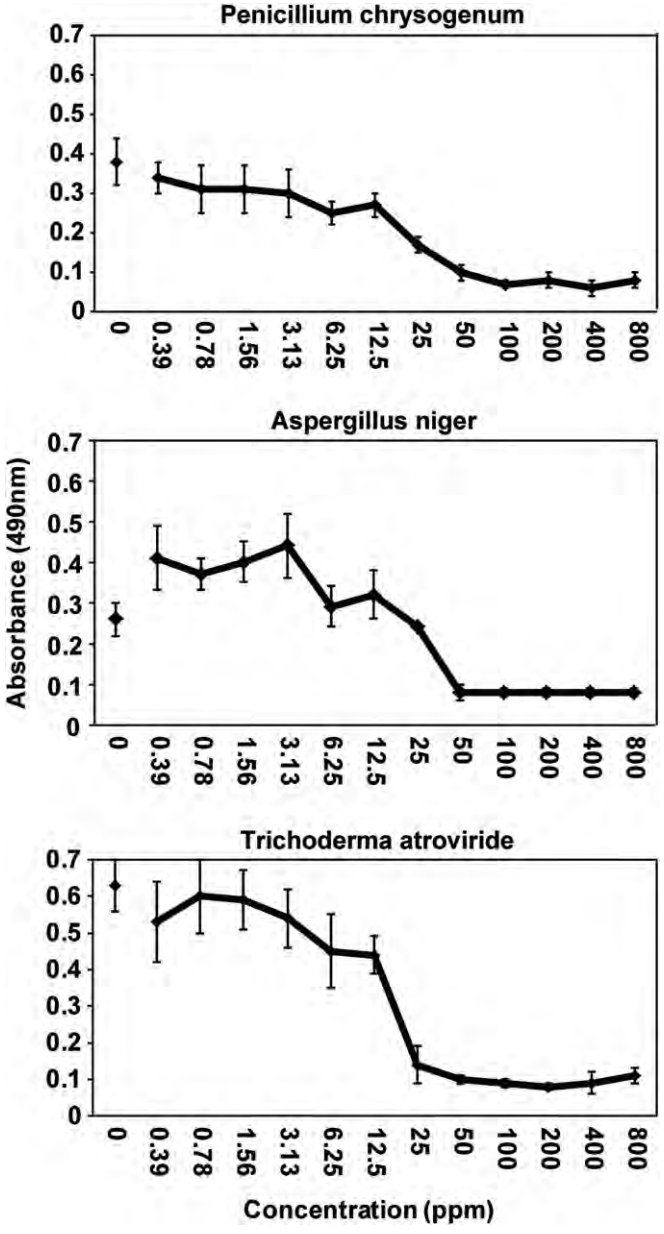

Fig. 1. XTT assay absorbance readings from mould spores exposed to a range of isothiazolinone concentrations ($n = 10$).

2.8. Fungicidal versus fungistatic activity

To determine if exposure to isothiazolinone temporarily or permanently inhibited spore germination, spores were pre-incubated with 50 ppm isothiazolinone for 24 h at 37 °C, washed twice with DI water as described in Section 2.7 and suspended in RPMI before testing for metabolic activity in the XTT assay. Following washing, spores were placed on 2% malt extract agar in a Petri dish and incubated at 27 °C for 2 weeks.

3. Results

Complete inhibition of mould growth using two standardized methods was compared with complete inhibition of metabolic activity in the XTT assay using isothiazolinone as the model mould inhibitor. Fig. 1 shows the average of 10 absorbance readings and standard deviations for mould spores from three test fungi exposed to two-fold dilutions of isothiazolinone. Absorbance at 490 nm is indicative of metabolic activity (i.e. mould spore germination). For all three test fungi, complete inhibition of metabolic activity occurred at dilutions containing ≥50 ppm isothiazolinone. Lower dilutions of isothiazolinone demonstrated decreasing levels of inhibition. Standard deviation in quantitative results for the XTT assay increased at concentrations of isothiazolinone that did not completely inhibit spore germination.

Comparative visual ratings for two standardized laboratory mould tests using vaccum-treated southern pine specimens are summarized in Table 1. Even though ASTM D4445 is a 4 week test and AWPA E24 is an 8 week test, incubation of test specimens was extended to 12 weeks to evaluate sustained inhibition. Average ratings and standard deviation for the four-week duration of this test are shown in bold text in Table 1. Ratings based on visual evaluations of mould growth can have a high standard deviation underscoring the importance of replicate specimens. Specimens treated with ≥50 ppm isothiazolinone substantially inhibited mould growth in the D4445 test over the 12-week duration of the test.

In the AWPA E24 test, specimens treated with ≥100 ppm isothiazolinone substantially inhibited mould growth for the 8-week duration of the test (bold values), but not for 12 weeks. Standard deviation for each treatment group was lower than that of the

Table 1
Average ratings for two standardized mould resistance tests on southern pine vacuum-treated with a range of isothiazolinone concentrations.

Test method	Isothiazolinone concentration (ppm)	Average mould ratings[a]		
		4 week	8 week	12 week
ASTM D4445	800	**0**	0	0
	400	**0**	0	0
	200	**0.1 (0.03)**	0.1 (0.3)	0.4 (0.4)
	100	**0.4 (0.5)**	0.2 (0.4)	0.3 (0.5)
	50	**0.1 (0.5)**	0.2 (0.4)	0.4 (0.7)
	25	1.0 (1.0)	0.4 (0.7)	0.6 (1.0)
	0	3.2 (1.6)	3.3 (1.4)	3.7 (1.1)
AWPA E24	800	**0**	**0**	1.4 (1.0)
	400	**0**	**0**	1.3 (1.1)
	200	**0**	**0**	1.6 (0.7)
	100	**0.1 (0.3)**	**0.3 (0.7)**	1.1 (1.1)
	50	1.4 (1.0)	4.6 (0.7)	5.0
	25	2.0 (1.4)	4.6 (0.7)	5.0
	0	2.3 (1.8)	4.9	5.0

Bold values indicate nearly complete mould inhibition for the normal duration of the ASTM method (4 week) or the AWPA method (8 week). Standard deviation is in parentheses.

[a] Value is the average rating of ten specimens per concentration. Rating scale: 0 = no growth, 1 = 20%, 2 = 40%, 3 = 60%, 4 = 80%, and 5 = 100% mould coverage.

D4445 test method. As with the ASTM method, the visual rating system is subjective and a high number of replicate specimens per treatment group is important for precision and reproducibility.

Results from the ATP assay corroborated the XTT results by verifying complete inhibition of metabolic activity after exposure of mould spores to 50 ppm isothiazolinone. There was no growth of spores from individual test fungi placed on malt extract agar following 24-h exposure to 50 ppm isothiazolinone and aseptic washing with DI water. This result suggests that isothiazolinone was fungicidal rather than fungistatic against spores from the test fungi under the conditions used in this study.

4. Discussion and conclusions

Despite micro- to macro- differences in the three test methods compared in this study, a critical factor for a screening method is the estimated minimal inhibitory concentration (MIC_{90}) for the chemical being evaluated. The estimated MIC_{90} for isothiazolinone for the XTT assay and D4445 test are in agreement ($\geq$50 ppm), and are within one (two-fold) dilution from the estimated MIC_{90} for isothiazolinone in the E24 method ($\geq$100 ppm). Such screening assays generate relative comparisons of mould inhibitors and are subject to some degree of biological variability. A higher MIC_{90} in the E24 test might also be expected due to specimen exposure to a greater variety of air and soil microbes in a non-sterile test apparatus compared to the monocultures utilized in the other two methods. Higher standard deviations seen at fungistatic concentrations of biocide would be anticipated due to the sensitivity of the assay to mitochondrial dehydrogenase activity (Levitz and Diamond, 1985; Hawser et al., 1998) and inherent metabolic variability in spore germination rates.

As a screening tool for mould inhibitors, the XTT assay offers several advantages over the current standardized methods. Quantitative results can be obtained in just 2 days without the need to treat and condition wood specimens saving time and resources.

The redeeming feature of the ASTM test is the low cost for disposable Petri dishes and the polyethylene spacer. It is also a relatively simple test to conduct and can test either monocultures or defined mixed cultures, but results are subjective. The AWPA method tests for resistance to polycultures, but results are subjective. Materials to fabricate environmental chambers for the AWPA test are initially costly and chambers are cumbersome to clean between tests. Specimens in the environmental chamber are susceptible to possible influence of volatiles from adjacent samples so multiple chambers are needed to randomize specimens and/or isolate volatile treatments. Overall, the quantitative colorimetric XTT micro-assay is rapid, sensitive and results correlate well with current standardized test methods.

References

Altman, F.P., 1976. Tetrazolium salts and formazans. Progress in Histochemistry and Cytochemistry 9, 1–56.

American Society for Testing Materials (ASTM), 2010. Standard test method for fungicides for controlling sapstain and mould on unseasoned lumber (laboratory method). ASTM D4445–10, West Conshohocken, PA. In: Annual Book of Standards, vol. 04.10, pp. 496–500.

American Wood Protection Association Standards (AWPA), 2010. Standard method of evaluating the resistance of wood product surfaces to mould growth. E24–06. Birmingham, AL. In: AWPA Book of Standards, pp. 440–443.

Clausen, C.A., Yang, V.W., 2011. A rapid Colorimetric Assay for Mould Spore Germination Using XTT Tetrazolium Salt. International Research Group on Wood Protection, Stockholm, Sweden. Publication IRG/WP 11–20462.

Hawser, S.P., Norris, H., Jessup, C.J., Ghannoum, M.A., 1998. Comparison of a 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide (XTT) colorimetric method with the standardized National Committee for Clinical Laboratory Standards methods of testing clinical yeast isolates for susceptibility to antifungal agents. Journal of Clinical Microbiology 36, 1450–1452.

Kuhn, D.M., Balkis, M., Chandra, J., Mukherjee, P.K., Ghannoum, M.A., 2003. Uses and limitations of the XTT assay in studies of *Candida* growth and metabolism. Journal of Clinical Microbiology 41, 506–508.

Levitz, S.M., Diamond, R.D., 1985. A rapid colorimetric assay of fungal viability with the tetrazolium salt MTT. Journal of Infectious Disease 152, 938–945.

Meletiadis, J., Mouton, J.W., Meis, J.F.G.M., Bouman, B.A., Donnelly, J.P., Verweij, P.E., 2001. EUROFUNG Network Colorimetric assay for antifungal susceptibility testing of *Aspergillus* species. Journal of Clinical Microbiology 39, 3402–3408.

Moore, G.E., Gerner, R.E., Franklin, H.A., 1967. Culture of normal human leukocytes. Journal of the American Medical Association 199 (8), 519–524.

National Committee for Clinical Laboratory Standards (NCCLS), 1997. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast. Approved Standard M27-A.

Pierce, C.G., Uppuluri, P., Tristan, A.R., Wormley, F.L., Mowat, E., Ramage, G., Lopez-Ribot, J.L., 2008. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nature Protocols 3, 1494–1500.