Journal of Microbiological Methods 145 (2018) 10–13

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

Note

Evaluation of colorimetric assays for determination of H_2O_2 *in planta* during fungal wood decomposition

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ARTICLE INFO

Keywords:
Brown rot
Fungi
Amplex red 10
Radical

ABSTRACT

Hydrogen peroxide (H_2O_2) plays a critical role in generating oxygen radicals as fungi attack and deconstruct plant cell walls. Its concentrations *in planta*, however, are often low during decomposition and evade detection by traditional methods. Here, we compared relevant methods and selected the best based on detection limits and selectivity.

Wood-degrading fungi harness oxidative chemistry to degrade lignin and metabolize carbohydrates in plant cell walls. As a specific example, brown rot-type fungi such as *Postia placenta* and *Gloeophyllum trabeum* use Fenton based oxidative radicals during wood decay, with H_2O_2 proposed as one of the oxidants involved in the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow OH^- + Fe^{3+} + \cdot OH$) (Goodell et al., 2008; Martinez et al., 2009). Various efforts have been made to track extracellular H_2O_2 content generated during wood decay, including brown rot, but the lack of a selective assay has hampered progress (Green and Highley, 1997). By using the catalase-aminotriazole method, Koenigs (1974) observed the H_2O_2 production coupled with cellulose depolymerization, but it was subsequently demonstrated to be an invalid method (Highley, 1981). More recently, (Kim et al., 2005) used cytochemical techniques to infer H_2O_2 produced *in planta* by *Tyromyces palustris* and *Coniophora puteana*, showing diffusion of H_2O_2 into the wood cell wall in early wood decay using Transmission Electron Microscopy (TEM). This technique, however, is expensive and time-consuming, and it was not designed for general comparative assays.

Instead of measuring H_2O_2, many studies have since opted to detect H_2O_2-generating enzymes, their coding transcripts (Ferreira et al., 2015; Zhang et al., 2016; Zhang and Schilling, 2017), or the associated radicals generated by the Fenton reaction (Kaneko et al., 2005). These approaches have indirectly implicated H_2O_2 in various key decomposition pathways, but they remain putative and lacking concentration data that might provide a more direct mechanism and enable reaction modeling.

Given this analytical deficit, we selected four methods previously reported as sensitive for quantifying low concentrations of H_2O_2 in biological samples (Li et al., 2017; Ritschkoff and Viikari, 1991; Wang et al., 2017; Zhou et al., 2006), with the goal of tracking extracellular H_2O_2 during wood decay by a model brown rot fungus *Postia placenta* (MAD 698-R) (Fig. 1). To do this, we extracted extracellular H_2O_2 from brown-rotted aspen wood (*Populus. sp*) obtained as described by Zhang et al. (2016). Wood sections ($n = 12$) along directionally-colonized thin wood wafers were pooled to represent no decay, early stage decay, and late stage decay (Table 1). These were extracted with 50 mM citrate buffer pH 3.3 or organic solvents $CHCl_3$:CH_3OH (2:1), with a ratio of 1:3 for sample weight (g): buffer (ml). Five minutes of vacuum, followed by 90 min shaking at 180 rpm and 4 °C, and centrifugation at 4000 rpm and 4 °C for 7 min completed the extraction. The supernatants were collected for H_2O_2 quantification.

There were four relevant H_2O_2-measuring methods used in this work. First, 4-AP method was applied as previously described by Zhou et al. (2006). Briefly, equal volumes of extract and colorimetric reagent (0.2 mg/ml 4-AP, 0.2 mg/ml phenol and 44 units/ml HRP in 100 mM acetate buffer, pH 5.6) were mixed, followed by incubation at 35 °C for 10 min, and absorbance measurement at 505 nm (Varian Cary 50 Bio UV–Visible spectrophotometer). Second, FOX method was applied as Setyo et al. (2010). Briefly, 900 µL of FOX reagent [25 mM H_2SO_4, 880 mg/L butylated hydroxytoluene, 76 mg/L XO, and 98 mg/L $(NH_4)_2Fe(SO_4)_2.6H_2O$ in methanol] were added to 100 µL of sample, followed by incubation at 25 °C for 30 min, and absorbance measurement (560 nm). Third, ABTS method was applied as described by Woo et al. (2003). Briefly, 130 µL of extract, 275 µL of 100 mM acetate

Abbreviations: 4-AP, 4-aminoantipyrine; ABTS, 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); FOX, ferrous oxidation - xylenol orange assay; XO, xylenol orange; AR10, amplex red 10; HRP, horseradish peroxidase; LOD, limit of detection; LOQ, limit of quantification

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https://doi.org/10.1016/j.mimet.2017.12.004
Received 7 November 2017; Received in revised form 8 December 2017; Accepted 9 December 2017
Available online 11 December 2017
0167-7012/

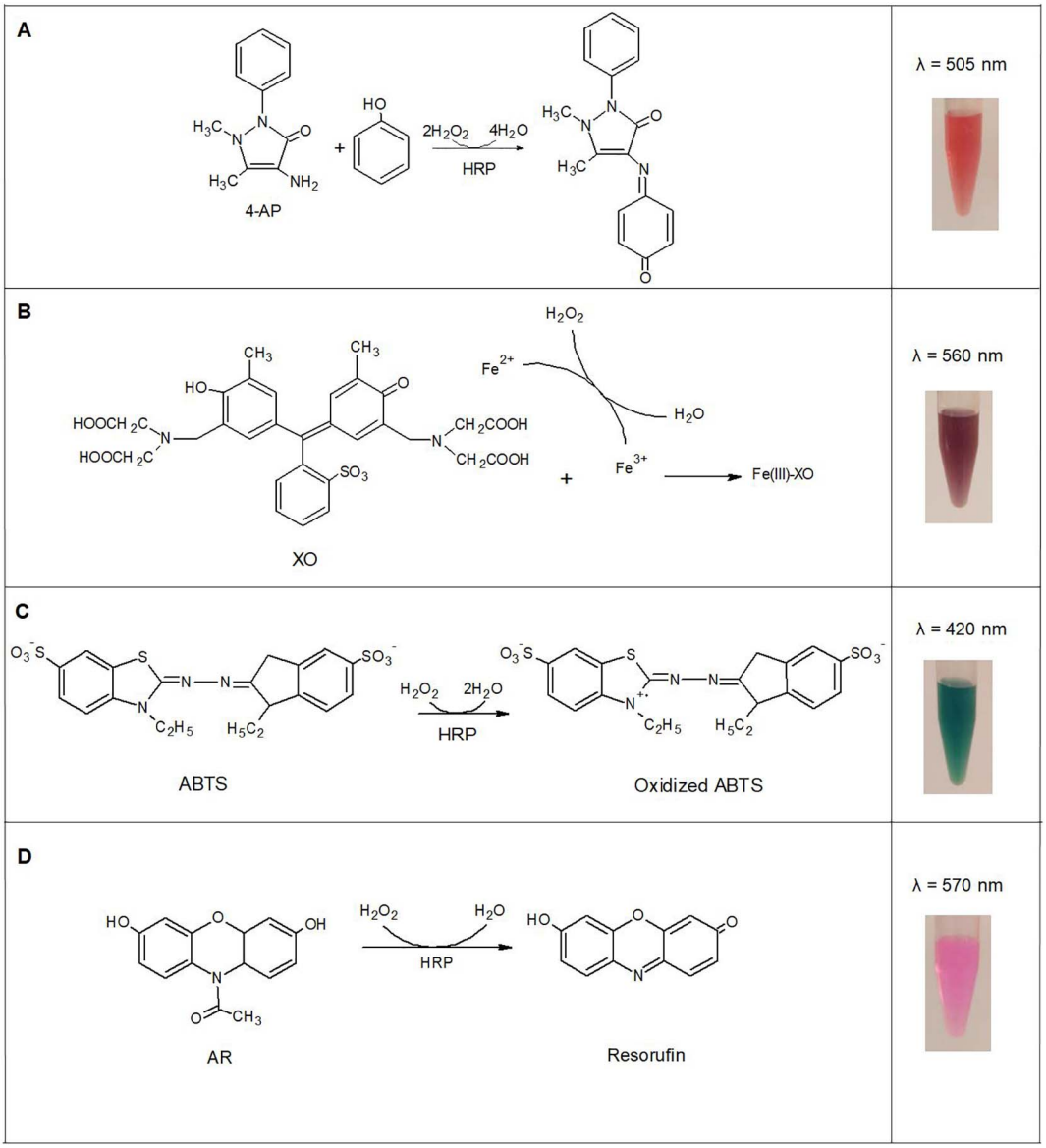

Fig. 1. Fundamental reactions and product detection wavelengths for H_2O_2 quantification methods. (A) 4-AP reacts with phenol and H_2O_2 as an oxidizing agent catalyzed by horseradish peroxidase (HRP) to produce a chromogenic adduct that can be detected at 505 nm. (B) H_2O_2 oxidizes Fe(II) to Fe(III), which in turn forms a chromogenic complex (λ = 560 nm) when is chelated by Xylenol Orange dye (XO). (C) ABTS is oxidized by H_2O_2 in the presence of HRP, producing the cation radical $ABTS^{\cdot+}$ that presents light to dark green color with strong absorbance at 420 nm. (D) AR-10 is oxidized by H_2O_2 in the presence of HRP, producing the resorufin molecule, which presents a pink color and absorbs strongly at 570 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

buffer pH 5.6, 200 µL of 5 mM ABTS, and 200 µL of 275 units HRP were mixed and incubated at room temperature for 30 min, followed by absorbance measurement (420 nm). Fourth, AR10 method was applied according to the manufacturer instructions (Invitrogen). Briefly, equal volumes of extract and working solution (0.1 mM AP10 and 1 units HRP in 50 mM phosphate buffer pH 7.4) were mixed, followed by incubation at room temperature for 30 min, and absorbance determination (570 nm). In all cases, the blank was the extract incubated at 25 °C for 1 h with 2990 units of catalase for removing H_2O_2.

The preparation processes for extracting H_2O_2 from brown-rotted wood were evaluated, mainly considering two H_2O_2 destabilizing sources: 1) H_2O_2-consuming enzymes, and 2) minerals. With respect to the first source, two different options were assayed but with undesirable results. First, boiling the extracts was tried in order to inactivate the H_2O_2-degrading enzymes. However, critical degradation was observed for the boiled H_2O_2 standards at low-concentrations (< 20 µM), which rendered this option inadequate for quantifying lower amounts of H_2O_2. Second, extraction using organic solvents $CHCl_3$:CH_3OH (2:1) was considered to inactivate the H_2O_2-consuming enzymes, as applied by Papapostolou et al. (2014). Notwithstanding, the application of this methodology to solid-state culture showed to be prohibitive given the difficulty to discriminate interferences of intracellular H_2O_2 released by organic disruption. Finally, we decided to extract H_2O_2 with an aqueous buffer rather than organic solvents, and then immediatly measure the contents afterwards, by which the degrading effects of catalase in extracts were minimized relative to the long term decay process (i.e., 30–35 mm section equals 13–15 days decay).

Another source that can destabilize H_2O_2 in the samples is the presence of minerals such as iron or manganese (Baciocchi et al., 2004), which are accumulated particularly in old fungal solid-state cultures (Jellison et al., 1997). Watts et al. (2007) and Schmidt et al. (2011) demonstrated that the presence of organic acids such as phytate and citrate can stabilize H_2O_2 up to two orders of magnitude. Accordingly, a citrate buffer was used here to avoid mineral-catalyzed decomposition of H_2O_2 in the fungal extracts.

Calibration curves for H_2O_2-quantifying methods were generated,