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Oxidative stress and culture atmosphere effects on bioactive compounds and laccase activity in the white rot fungus *Phlebia radiata* on birch wood substrate

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ABSTRACT

Wood-decaying white rot fungi live in changing environmental conditions and may switch from aerobic to fermentative metabolism under oxygen depletion. Decomposition of wood and lignocellulose by fungi is dependent on enzymatic and oxidative biochemistry including generation of reactive oxygen species. In this study, we subjected semi-solid wood-substrate cultures of the white rot fungus *Phlebia radiata* to oxidative stress by addition of hydrogen peroxide under aerobic and anaerobic cultivation conditions. Wood decomposition and fungal metabolism were followed by analysis of extracellular organic compounds, mycelial growth, and laccase activity. Under both atmospheric conditions, accumulation of bioactive aromatic compounds from birch wood occurred into the culture supernatants after hydrogen peroxide treatment. The supernatants inhibited both fungal growth and laccase activity. However, the fungus recovered from the oxidative stress quickly in a few days, especially when cultivated under regular aerobic conditions. With repeated hydrogen peroxide treatments, laccase suppressive-recovery effect was observed. Culture supernatants demonstrated antioxidant and antimicrobial effects, in concert with emergence of chlorinated birch-derived organic compounds. Bioactivities in the cultures disappeared in the same pace as the chlorinated compounds were transformed and de-chlorinated by the fungus. Our results indicate tolerance of white rot fungi against excessive oxidative stress and wood-derived, growth-inhibiting and harmful agents.

1. Introduction

Saprotrophic fungi of Basidiomycota demonstrate different strategies in their extracellular decomposition of plant biomass and lignocellulosic material (Eastwood et al., 2011; Floudas et al., 2012; Lundell et al., 2014; Boddy and Hiscox, 2016). For the wood-decaying fungi of Agaricomycetes, extracellular oxidative conditions are essential in the degradative processes of white rot (mainly enzymatic decomposition) and brown rot (mainly non-enzymatic Fenton-chemistry) (Hammel et al., 2002; Eastwood et al., 2011; Floudas et al., 2012; Arantes and Goodell, 2014). In the extracellular enzymatic and non-enzymatic degradative reactions, production and involvement of reactive oxygen species (ROS), especially hydrogen peroxide, is fundamental (Mattila et al., 2022).

Supply of hydrogen peroxide is necessary for the action of lignin-modifying peroxidases produced by the white rot fungi (Hofrichter et al., 2010; Lundell et al., 2014; Bissaro et al., 2018) and for the non-enzymatic Fenton chemistry created by brown rot fungi for decomposition of wood polysaccharides, particularly cellulose (Hammel et al., 2002; Eastwood et al., 2011; Castaño et al., 2018; Shah et al., 2018). Alongside the extracellular decomposition reactions reactive oxygen species are involved in several functions in fungi such as cellular communication and interaction with other fungi (Tornberg and Olsson, 2002; Eyre et al., 2010), microbes and host organisms, and produced as a defense agent (Boddy and Hiscox, 2016; Mattila et al., 2022).

To survive under continuous production of reactive oxygen species and oxidative conditions in their environments, fungi demonstrate various protection responses and concerted enzyme activity pathways (Mattila et al., 2022). Some of the tactics include spatial and temporal regulation in cellular functions, expression of H_2O_2 producing, quenching, and tolerating enzymes and production of antioxidant compounds. Many of these mechanisms have been studied in

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wood-decaying brown rot fungi (Hammel et al., 2002; Arantes and Goodell, 2014; Castaño et al., 2018; Shah et al., 2018) whereas in white rot fungi, the focus has been more on the activities of specific ROS utilizing and quenching enzymes (Mattila et al., 2022) rather than on fungal metabolic response to oxidative stress.

Reactivity of white rot fungal isolated redox enzymes with O_2 (oxidases and laccases) and H_2O_2 (peroxidases and peroxygenases) has been thoroughly studied (reviewed in Lundell et al., 2010; Hofrichter et al., 2010; Bissaro et al., 2018). It has been shown that production of laccase and manganese peroxidase in white rot fungi can be induced and enhanced by addition of hydrogen peroxide (Wiberth et al., 2019). However, oxidative conditions are often harmful towards the enzymes (Castaño et al., 2018, 2021). Responses to hydrogen peroxide supplementation are dose dependent and may vary between fungal species from enhancing to inhibiting effects (Wiberth et al., 2019).

Wood-decaying white rot fungi are potential organisms for consolidated bioprocessing of wood-based and plant biomass waste materials to biofuels and other added-value products due to their ability of biodegradation of lignocellulose under aerobic and fermentative metabolism (Mattila et al., 2017, 2020). Not much is known yet, however, on the regulation of metabolism or enzyme activities produced by the fungi under changing atmospheric and environmental stress conditions.

Aim of this study was to investigate oxidative stress effects on mycelial growth and bioconversion activity of the white rot fungus *Phlebia radiata* in semisolid cultivations on birch wood sawdust. Oxidative stress was created by addition of hydrogen peroxide onto the fungal cultures kept at varying atmospheric conditions (aerobic or fermentative anaerobic). Focus was on the production of laccase enzyme activity, primary metabolites, and bioactive compounds. The oxidative treatment elicited a strong effect on fungal cultures under both cultivation atmospheres, together with accumulation of birch-wood derived compounds demonstrating antioxidant, antimicrobial and antifungal activities. Fungal mycelium apparently recovered from the oxidative treatment within a few days which was demonstrated by re-production of laccase activity under aerobic conditions, and ethanol under fermentative conditions. These findings demonstrate robustness of the fungus against oxidative stress, as well as potentiality of the fungal semisolid cultures for production of wood-based bioactive compounds.

2. Materials and methods

2.1. Set-up of the birch sawdust culture flasks

The Basidiomycota white rot fungus *Phlebia radiata* isolate 79 (FBCC 0043) (Kuuskeri et al., 2016; Mäkinen et al., 2019) was cultivated on silver birch (*Betula pendula*) sapwood sawdust in semisolid laboratory cultivations. Oven dried birch sawdust was sieved (less than 4.8 mm particle size) and added into 250 ml volume glass Erlenmeyer flasks each containing 10 g in dry weight of the sawdust. After autoclaving (121 °C, 15 min, dry run), 100 ml of sterile 1 % (w/vol) yeast extract (Neogen Europe Ltd) solution (autoclaved 121 °C, 15 min, 1 atm) was added into each flask.

2.2. First cultivation under fermentative conditions

To start the cultivation, three pieces of 6 mm diameter agar plugs from 4-day mycelial culture on malt extract (2 % (w/vol), Neogen Europe Ltd) agar (MEA medium) of the fungus were added in the flasks, on top of the sawdust layer. Four flasks were left un-inoculated representing the no-fungus control flasks (NF). The flasks were closed with cellulose stoppers and metal caps to allow gas exchange and aerobic cultivation conditions, and placed in an incubator at 25 °C without agitation. After five days of aerobic cultivation, tight rubber plugs with valved inlets allowing sample withdrawal and gas exchange were installed onto each flask. The rubber plugs were kept on until the end of the cultivation to ensure establishment of fermenting (oxygen depleted), "anaerobic" cultivation conditions, and control of the culture atmosphere (Mattila et al., 2017, 2020). One of the sampling valve inlets included tubing extending directly through the substrate matrix to the bottom of the flask. This allowed access to the culture fluid (water dissolved liquid phase, culture supernatant) and sampling without opening the flasks. Liquid samples of the culture supernatant of 0.5 ml were drawn from each flask with sterile plastic syringes through the sampling valve.

2.3. Addition of H_2O_2 into the cultures

Oxidative treatment was applied to the culture flasks through the inlet valves three times during the 70-day long experiment on time points 10, 24 and 38 days of cultivation. Four parallel replica cultures flasks were subjected for each of the treatments and cultivation atmospheres. Aliquots of either sterile ultrapure water or H_2O_2 were added to final 0.1 % (w/v) (35 mM) concentration in the total volume of 100 ml of culture supernatant. H_2O_2 solution was made from 30 % highest purity grade H_2O_2 (Sigma Aldrich) into sterile ultrapure water each time prior to use. Air flushing into the AN+Air fungal cultures was done by flushing each flask with at least 5x air-space volume (5 × 200 ml) with membrane filtered air through the valve inlet of the flask sealing rubber plug. At the end of the experiment (on day 70) the culture supernatant and the solid materials were separated by filtering with clean rayon-polyester fabric (Miracloth, Calbiochem, Merck-Millipore). Both the liquid and solid material were stored at −20 °C for further analyses. pH of the culture supernatant samples was measured by using a standard coupled electrode (SI Analytics N 6000 BNC) and Orion Model 920A advanced pH/ISE meter.

2.4. Second cultivation under aerobic and anaerobic conditions

Second cultivation series was set up similarly but including both fermentative "anaerobic" and ordinary aerobic cultivation atmospheric conditions, together with and without hydrogen peroxide treatment. Three parallel culture flasks for sampling at five time points (days 5, 10, 11, 13 and 17) during the cultivation were set up for each treatment series. To make the substrate more acidic prior to inoculation with the fungus, yeast extract solution was adjusted to pH 3 with hydrochloric acid before autoclaving and addition into each flask containing the dry-autoclaved birch sawdust. Acidification of culture fluid was considered to promote fungal degradation and conversion of the wood substrate (Mattila et al., 2020). Fungal cultures were inoculated with 3 pieces of 6 mm diameter plugs from 8-day mycelium grown on MEA medium. In total, 15 birch-wood substrate flasks were left un-inoculated to serve as no-fungus controls (NF2). All flasks were initially closed with cellulose stoppers and metal caps and incubated at 25 °C without agitation. On cultivation day five, rubber plugs with inlet valves were installed to allow the fermentative "anaerobic" conditions both in the fungal cultures (AN2 cultures) and no-fungus controls (NF2 flasks). Half of the fungal cultures were kept as such (without change of the stoppers) under aerobic conditions (AE2 cultures). On cultivation day 10, after sample taking, 9 flasks from anaerobic and aerobic series, respectively, and 9 no-fungus control flasks were treated with H_2O_2 to a final 0.1 % (w/v) concentration (AN2+H2O2, AE2+H2O2, and NF2), similarly as in the first cultivation series. In the rest of the fungal cultures (AN2, AE2), sterile ultrapure water was added instead of H_2O_2.

Sampling at each time point was done by fully emptying three parallel culture flasks from each of the treatment series. Culture fluid (culture supernatant) was separated from the solid materials by filtering through fabric (Miracloth, Calbiochem, Merck-Millipore). Both the liquid samples and solid materials were separately stored at −20 °C for further analyses. At the last three sampling timepoints (days 11, 13 and 17) one of the three parallel culture flasks from each treatment series was randomly selected for estimation of mycelial biomass (mycelial growth). The hyphal mat on top was detached from the wood sawdust

Table 1
Q-TOF instrument parameters applied in the detection of aromatic compounds.

Ion mode	Capillary voltage (kV)	Source temperature (°C)	Sampling cone	Source offset	Desolvation temperature (°C)	Desolvation gas flow (liter/h)	Nebulizer gas flow pressure (bar)	Ion energy	Trap collision energy	Ion detection range (*m/z*)
+	2.5	120	20.0	60.0	600	1000	6.0	1.0	4.0	50–2000
–	2.0	120	25.0	80.0	600	1000	6.0	1.8	30.0	50–2000

substrate material, weighed fresh and stored at −20 °C.

2.5. Enzyme activity assays

Laccase and manganese peroxidase (MnP) enzyme activities were measured from the culture supernatant samples according to previously adopted methods (Rytioja et al., 2014; Kuuskeri et al., 2015; Mali et al., 2019) using a multimode microplate reader (Spark, Tecan, Switzerland) and clear polystyrene 96-well microplates. Laccase activity was monitored as increase in absorbance at 420 nm to follow formation of the radical product of the substrate compound ABTS [(2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] (Lundell and Hatakka, 1994). MnP activity was determined as increase in absorbance at 270 nm representing formation of the Mn^{3+} malonate complex (Hofrichter et al., 2010; Kuuskeri et al., 2015). Enzyme reactions were monitored in three technical replicate reactions for each culture flask sample (3–4 biological replicate flasks at each time point). Negligible activity of less than 0.01 nkat/ml observed in individual culture samples and cultivation timepoints were considered as 0 value in the analysis. Mean value with deviation of the biological replica cultures (n = 3–4) and statistical significance were calculated, the latter with the 2-tailed student's T-test assuming equal variance using Microsoft Office Excel software.

For laccase inhibition assays, a high-laccase activity *P. radiata* culture medium supernatant reference sample from a previous cultivation was used to determine the potential laccase enzyme inhibitory activity of compounds present in the lyophilized culture supernatants (LS) (Appendix A). Activity of the reference sample was measured alone and after mixing with either ultrapure water, 70 % (vol/vol) methanol or aliquots of the lyophilized supernatants in a ratio of 40 µl laccase reference enzyme + 10 µl of tested sample or solvent. Pooled LS aliquots from parallel replica cultures were tested. Effect of hydrogen peroxide on laccase activity was determined by mixing the reference enzyme with H_2O_2 to yield a 0.1 % (w/v) final H_2O_2 concentration, similar to the treatment of the fungal cultures. The reaction mixtures were incubated 30 min at room temperature before measurement of laccase activity using the microplate method and ABTS as substrate (see above). Enzyme reactions were monitored for a prolonged period of 30 min to see the effect of possible competing substrate compound inhibition even after occasional lag phase of product formation.

2.6. HPLC analysis of fungal fermentation metabolites

Extracellular water-soluble organic compounds (sugars, alcohols, organic acids) produced from the birch wood sawdust substrate in the second cultivation series were analysed by using the previously adopted HPAE-PAD method (Mattila et al., 2020). Waters Alliance e2695 HPLC separation module was connected to both PDA (photo-diode array) detector and Waters 2414 RI (refractive index) detector, which was operated at sensitivity value 128. In each run, filtered (through Whatman Uniflo 0.2 µm regenerated cellulose filter units, Cytiva) culture supernatant samples were injected in 20 µl into the mobile phase and eluted with isocratic 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. Agilent Hi-Plex H (300 × 6.5 mm) column equipped with guard column (50 × 7.7 mm) was used to separate the analytes at column temperature of 65 °C. For identification and quantification of the separated molecules, concentration series of commercially available reference compounds were made in the range of 0.05–2.0 g/liter including sugar monomers and dimers, acetic acid, ethanol, methanol, and glycerol (all highest purity grade). Detection limit was set to ½ of the lowest concentration (0.025 g/liter) of each reference compound analyzed and thereby, peaks falling at or below the limit were treated as zero values. As an internal standard for control of analytical quality and technical performance, 1-propanol in final concentration of 1 g/liter was added into each sample before injection.

2.7. UPLC-MS analysis of aromatic compounds

Analysis of aromatic compounds present in the lyophilized culture supernatants (LS samples) (Appendix A) was carried out using Waters SYNAPTG2-Si UPLC-PDA-QTOF equipment system. Methanolic LS samples were thoroughly mixed and filtered through 0.22 µm pore size PTFE syringe filters (Spheros, Lab Logistics Group GmbH). Organic compounds were separated using a 100 × 2.1 mm sized 1.7 µm C8 100 Å Kinetex (Phenomenex) column and reversed phase elution at 40 °C column temperature. Mobile phase consisted of two solvents: 0.1 % formic acid (vol/vol) in ultrapure water (solvent A) and 0.1 % formic acid (vol/vol) in acetonitrile (solvent B). All solvents were of HPLC grade. Filtered samples were injected in 2 µl into the mobile phase and run in 18 min with a changing mobile phase gradient (0 min 95 % A; 2 min 80 % A; 4 min 80 % A; 10 min 100 % B; 12 min 100 % B; 12.10 min 95 % A; 18 min 95 % A) with a constant flow rate of 0.4 ml/min. Quantification and identification of the aromatic compounds was performed with Waters Acquity PDA (photodiode absorbance) coupled with Q-TOF (quadrupole-time-of-flight) mass detection system using both positive and negative ion modes (Table 1).

Separated compound peaks were recognized and their areas were calculated from UV absorbance chromatograms by using the MassLynx mass spectrometry software (Waters) automatic annotation and integration tool with 100 kAU (arbitrary absorbance units) threshold value for the peaks. Ten peaks with the largest area emerging and disappearing in the chromatograms of LS samples taken from culture flasks before and after H_2O_2 treatment were taken into closer inspection. LOTUS database (Rutz et al., 2022) was used to aid compound identification.

2.8. Antioxidant activity assay

Antioxidant activity of the culture samples was assayed by measuring their capacity to reduce stable DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals in a methanolic solution in comparison to Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) reference compound standard curve. Results were presented as Trolox equivalent antioxidant capacity (TEAC) of each sample (Brand-Williams et al., 1995; Mau et al., 2004; Thaipong et al., 2006). Both the lyophilized culture supernatant (LS) and non-lyophilized culture supernatant (non-LS) samples solubilized in 70 % (vol/vol) methanol - 30 % ultrapure water mixture (Appendix A) were assayed in 96-well plastic microplates using the multimode reader (Spark, Tecan, Switzerland). Briefly, 180 µl of the LS or non-LS sample or the Trolox (EMD Millipore Corp., USA) reference compound in 70 % methanol solution were pipetted in the microplate wells as technical triplicates. The reactions were started by adding 20 µl of 1 mM DPPH (Alfa Aesar, Thermo Fisher Scientific) dissolved in 100 % methanol, and followed by decrease in absorbance at 517 nm at 22 °C for 25 min at 5 min intervals. After measurement the plates were tightly wrapped with 96-well plate sealing

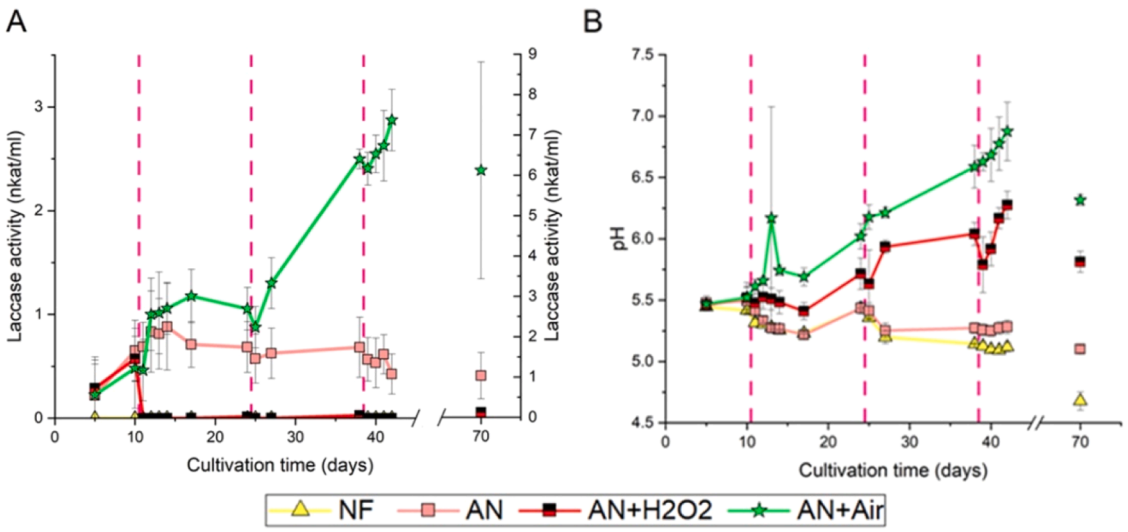

Fig. 1. Changes in A) production of laccase activity (nkat/ml), and B) pH in the culture supernatants of the first cultivation of *P. radiata* on birch wood sawdust under fermentative conditions. NF, no fungus; AN, anaerobic, fermentative fungal cultures; AN+H2O2, anaerobic cultures treated with hydrogen peroxide; AN+Air, anaerobic cultures treated with air. Dashed pink lines depict the three time points of the treatments. The mean value and standard deviation of four parallel replica cultures are shown for each series. In A) notice the different y scales for laccase activity between day 70 and the earlier cultivation days.

tape (Thermo Scientific) to prevent solvent evaporation, covered with aluminum foil and kept at 25 °C in the dark for a further 20 h before a second measurement. Additional 20 h incubation was adopted to allow potentially slowly reducing or reacting compounds in the samples to reach the reaction steady-state (Brand-Williams et al., 1995).

2.9. Antimicrobial activity assay

Antibacterial and antifungal activities of LS samples (lyophilized culture supernatants; Appendix A) were assessed with an antimicrobial disk agar diffusion assay based on the standard protocols (NCCLS, 2004; CLSI, 2012). Three bacterial species type strains, namely *Staphylococcus aureus* (HAMBI 66), *Pseudomonas aeruginosa* (HAMBI 25), *Escherichia coli* (HAMBI 1723), together with two clinically relevant fungal yeast species strains *Candida albicans* (HAMBI 484 / FBCC 2462) and *Candida tropicalis* (HAMBI 485 / FBCC 2463 / ATCC 750) were used in the assays. Test microbes were available from the Microbial Domain Biological Resource Centre HAMBI (part of the Biodiversity Collections Research Infrastructure in the Helsinki Institute of Life Sciences, University of Helsinki, Finland). Detailed description of the antimicrobial test protocol and tested samples are found as supplementary information (Appendix A).

2.10. Antifungal activity against Phlebia radiata

Antifungal activity of the LS samples (Appendix A) against *P. radiata* itself was assessed with a disk diffusion assay developed in this study for non-conidiating filamentous fungi. *P. radiata* (FBCC 0043) mycelium was cultivated at 25 °C in the dark on 2 % malt extract agar (MEA) plates for 7 days. Two plastic tissue culture flasks with ventilated caps (T175, Sarstedt) were filled with 75 ml of 2 % (w/vol) malt extract (Neogen) liquid medium (ME). The flasks were inoculated with 10 pieces of 6 mm diameter hyphal agar plugs from 7-day cultivated MEA plates. The fungal liquid ME cultures were kept statically (non-agitated) in a closed incubator at 25 °C. After 4 days of cultivation, mycelium and ME culture fluid were homogenized by aseptically transferring the full content of each culture flask into sterile 1-liter volume steel cup, closed with steel lid and mixed with laboratory blender (Waring Laboratory Science) 3 times in 15 second pulses at low rpm mode. Turbidity of the mycelial homogenate was measured as optical density at 620 nm against ME liquid medium with a benchtop colorimeter (Clormic, JP Selecta). Mycelial homogenate of OD_{620} value 1.25 was transferred in 500 µl volume on the surface of 2 % MEA medium in a larger petri dish (14 cm diameter), spread evenly with a sterilized glass triangle, and allowed to dry for 5–10 min lid ajar in a laminar flow hood. After drying, antifungal reference compounds in ready-made 6 mm diameter disks (nystatin 100 U/disk, fluconazole 25 µg/disk, Oxoid brand, Thermo Fisher Scientific) and blank 6 mm diameter, sterile paper disks (Oxoid brand, Thermo Fisher Scientific), on which the LS test samples were pipetted, were applied on the surface of the air-dried mycelial suspension on the MEA medium. NF samples (also catalase treated, Appendix A) were applied on the blank disks as 20 µl/2 stacked disks, while the other samples were applied as 37 µl/2 stacked disks. The closed plates were sealed with parafilm and incubated at 25 °C in the dark for 3 days in upright position. Hyphal growth of *P. radiata* was inspected, and inhibition zone diameter (IZD) values for the LS and NF samples and reference compounds were measured in millimetre (mm) scale accuracy.

3. Results

3.1. Laccase activity and pH in the first cultivation

Laccase activities in the fungal culture supernatant samples of the first fermentative cultivation series varied depending on the treatment (Fig. 1A). Flushing with air stimulated production of laccase activity (AN+Air cultures) in comparison to the non-aerated culture conditions (AN cultures). However, addition of hydrogen peroxide (AN+H2O2 cultures) quenched the activity almost completely. Until cultivation day 10, laccase activities were similar in all of the fungal cultures (approximately 0.5 nkat/ml). These moderate laccase levels endured in the non-treated fermentative fungal cultures (AN cultures), only slightly increasing to level of 1 nkat/ml on the last day 70 (Fig. 1A, series AN). Flushing the closed AN cultures with air resulted in stimulated production of laccase activity. This was especially distinguishable after the second treatment as enzyme activity steadily increased for several weeks, reaching 3 nkat/ml on day 42 and further doubling to 6 nkat/ml on day 70 (Fig. 1A, series AN+Air).

Addition of hydrogen peroxide into the fermentative fungal cultures, however, suppressed laccase activity (Fig. 1A, series AN+H2O2). One day after hydrogen peroxide treatment, laccase activity in the fermentative cultures dropped down and remained at zero or negligible levels until the end of the cultivation (Fig. 1A, series AN+H2O2). On day 70, very low laccase activity (0.12 ± 0.11 nkat/ml) was measurable in three

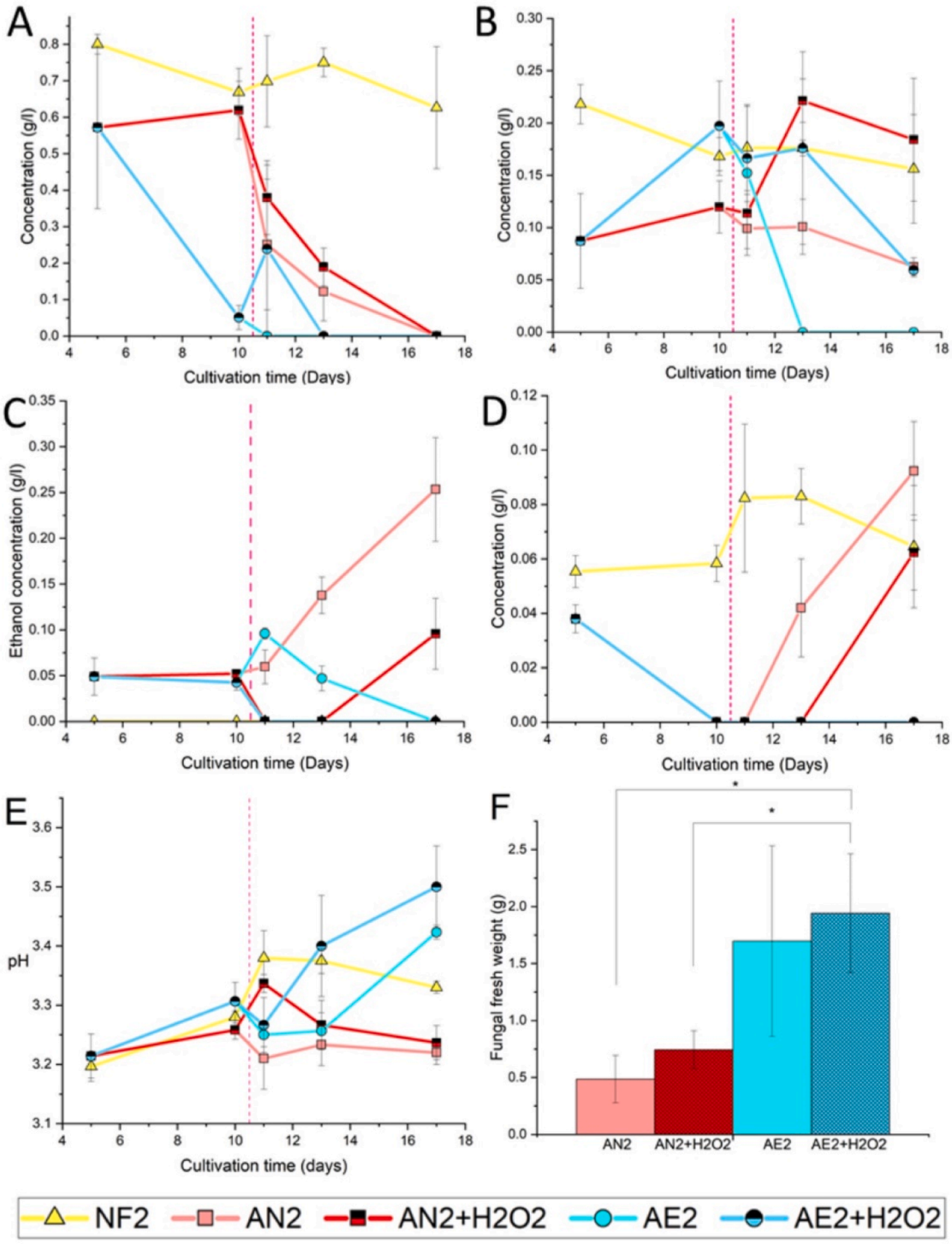

Fig. 2. Fungal metabolites, mycelial growth, and accumulation of sugars in the culture supernatants during the second cultivation. Concentration of detected compounds are presented in grams/liter. A) disaccharides, B) glucose, C) ethanol, D) acetic acid, E) pH, and F) mycelial fresh weight. NF2, no fungus control flasks; AN2, anaerobic, fermentative fungal cultures; AN2+H2O2, fermentative flasks treated with hydrogen peroxide; AE2, aerobic fungal cultures; AE2+H2O2, aerobic fungal cultures treated with hydrogen peroxide. Dashed pink lines depict the time of H_2O_2 addition. Note the difference in concentration scales (y axis) for the compounds. The mean value and standard deviation are presented; $n = 3$ for NF2 control series samples; for fungal culture series $n = 12$ on day 5, $n = 6$ on day 10, $n = 3$ on days 11, 13 and 17. In F) statistically significant differences ($p < 0.05$) between the treatments are marked with an asterisk.

of the four parallel replica cultures (Fig. 1, series AN+H2O2). In contrast to laccase production, no MnP activity could be detected in the culture samples (data not shown), which may be a consequence of presence of enzyme assay disturbing wood-derived compounds (Kuuskeri et al., 2015; Mali et al., 2017).

In the first cultivation series, acidity of the culture supernatants also varied between the treatments (Fig. 1B). The AN+Air and AN+H2O2 series followed a similar trend of decline of acidity, that is the rise in pH values through the cultivation, starting from the initial pH of 5.5 and ending at pH maxima of 6.9 and 6.3 on cultivation day 42, respectively. Loss of acidity during fungal growth is notable in these culture series, since in the non-treated fungal cultures (series AN), supernatants

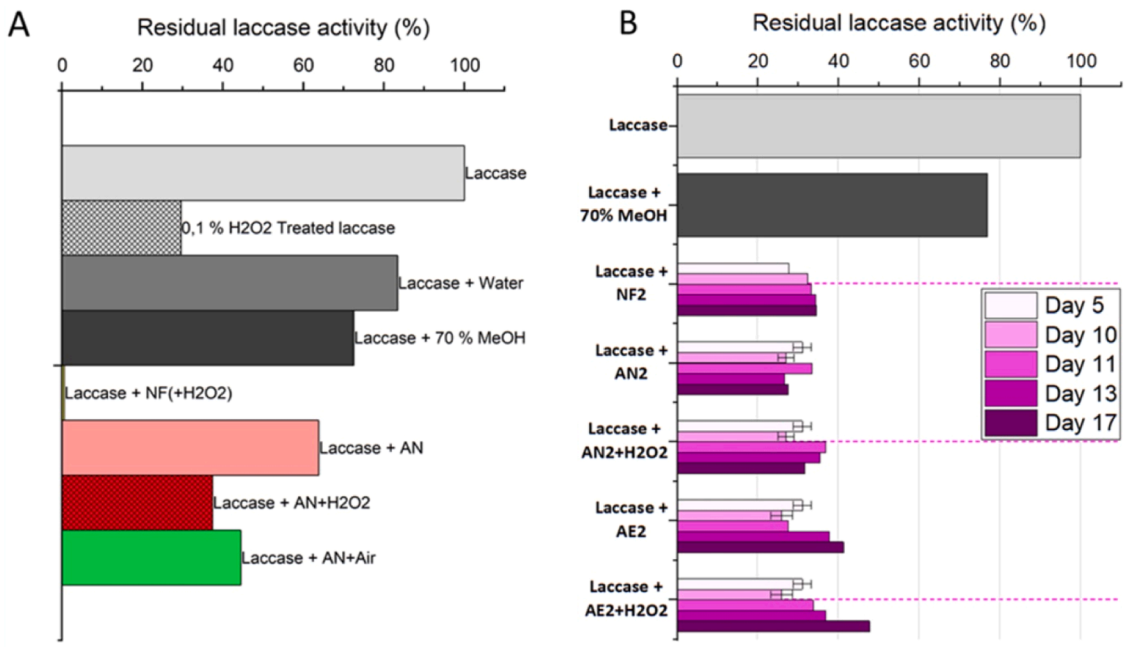

Fig. 3. *P. radiata* laccase activity after incubation with hydrogen peroxide or mixing with lyophilized culture supernatant samples. A) Laccase reaction mixtures containing 0.1 % H_2O_2 or the 70-day culture supernatant lyophilized end samples from the first cultivation series. Average of three technical replicates from pooled samples is depicted. Original laccase activity was 2.0 nkat/ml. NF, no fungus; AN, anaerobic, fermentative fungal cultures; AN+H2O2, anaerobic cultures treated with hydrogen peroxide; AN+Air, anaerobic cultures treated with air. B) Laccase mixed with 70 % methanol or lyophilized supernatants (sampling days illustrated with different shades of purple) of the second cultivation series. Mean values and standard deviation for days 5 and 10 of four and two pooled samples, respectively, and mean values for days 11, 13 and 17 of three technical replicates are depicted. Dashed pink lines depict the timing of hydrogen peroxide treatment into the cultures. Original laccase activity in this experiment was 2.6 nkat/ml. NF2, no fungus control flasks; AN2, anaerobic, fermentative fungal cultures; AN2+H2O2, fermentative flasks treated with hydrogen peroxide; AE2, aerobic fungal cultures; AE2+H2O2, aerobic fungal cultures treated with hydrogen peroxide.

became somewhat more acidic close to pH 5, in accordance with the no-fungus control flasks (NF series). In all cultures, end pH values on day 70 were 0.5–1.0 lower than measured on day 42. After hydrogen peroxide treatment, a drop in the pH was observed within one day, which was followed by a quick rise in a few days (Fig. 1B, series AN+H2O2). However, there was large variation between the parallel replica flask cultures after the treatments.

3.2. Metabolic activities in the second cultivation series

Fungal conversion of the birch wood substrate and production of extracellular metabolites was monitored in the second cultivation by HPLC analysis. Disaccharides, mainly trehalose present in the yeast extract, were found in the culture supernatants on day 5, detected as 0.8 g/liter in no-fungus control flasks (NF2 series) and 0.6 g/liter in the fungal cultures, but were quickly consumed by the fungus under aerobic cultivation conditions down to low concentrations on day 10 (0.05 g/liter, AE2 and AE2+H2O2 series, Fig. 2A). Under fermentative anaerobic conditions (AN2 and AN2+H2O2 series) the decrease in disaccharide concentration was less rapid leading to later exhaustion on day 17 of cultivation (Fig. 2A). Interestingly, treatment with hydrogen peroxide produced an extra pulse of disaccharides into the culture supernatants on day 11 in the aerobic fungal cultures (Fig. 2A, series AE2+H2O2).

Some glucose was also present in the culture medium already on day 5, also in the NF2 flasks (0.2 g/liter, Fig. 2B) whereas less than 0.1 g/liter glucose was present in the fungal cultures. In the aerobic cultures (AE2 series, Fig. 2B) glucose was consumed quickly following depletion of the disaccharides (Fig. 2A), falling below detection limit (0.025 g/l) on cultivation day 13 (AE2 series, Fig. 2B). Addition of H_2O_2 into the cultures, however, somewhat decelerated glucose consumption, and detectable levels of glucose were present until the end of cultivation on day 17 (AE2+H2O2 and AN+H2O2 series, Fig. 2B). Under fermentative conditions (series AN2) glucose was maintained at constant levels staying near to 0.1 g/liter for two weeks.

Highest ethanol concentrations were detected in the fermentative anaerobic fungal cultures accumulating to levels of 0.25 g/liter on cultivation day 17 (AN2 series, Fig. 2C), with an increasing trend of production following cultivation day 11. This is an indication of fungal fermentative metabolism (Mattila et al., 2017; Mäkinen et al., 2018). Production of ethanol (Fig. 2C) followed consumption of disaccharide sugars (Fig. 2A) in the AN2 fermentative fungal cultures. Small concentrations of ethanol were detected in all fungal cultures on days 5 and 10 (Fig. 2C), suggesting that the fungal mycelium also in the aerobic culture may have experienced partial oxygen depletion and turned to fermentation metabolism. Addition of hydrogen peroxide quickly abolished ethanol from the fungal cultures irrespective of the incubation atmosphere, either aerobic (series AE2+H2O2) or fermentative (series AN2+H2O2) (Fig. 2C). Fermentative state was recovered within a few days after the treatment leading to a second round of ethanol production in AN2+H2O2 cultures.

It is notable that low concentration of acetic acid was observed continuously in the no-fungus control flasks (series NF2, Fig. 2D), which may indicate release of acetate from the birch wood substrate (from acetylated hemicellulose sugar units) or the medium yeast extract solution. Acetate disappeared quickly by day 10 in the fungal cultures, and after addition of H_2O_2 (Fig. 2D). Appearance of low acetate concentrations in fermentative fungal cultures during the later stage of the cultivation, however, followed the pattern of ethanol formation (series AN2 and AN2+H2O2, Fig. 2C,D).

In the second cultivation, level of acidity in the fungal cultures was kept around the initially adjusted pH 3, namely below pH 3.5 in the aerobic cultures (series AE2 and AE2+H2O2) and around pH 3.2 in the fermentative fungal cultures (series AN2 and AN2+H2O2) (Fig. 2E). Regarding fungal mycelial growth, only the hydrogen peroxide treated aerobic fungal culture (AE2+H2O2 series) was significantly ($p < 0.05$) different (higher mycelial biomass production) in comparison to

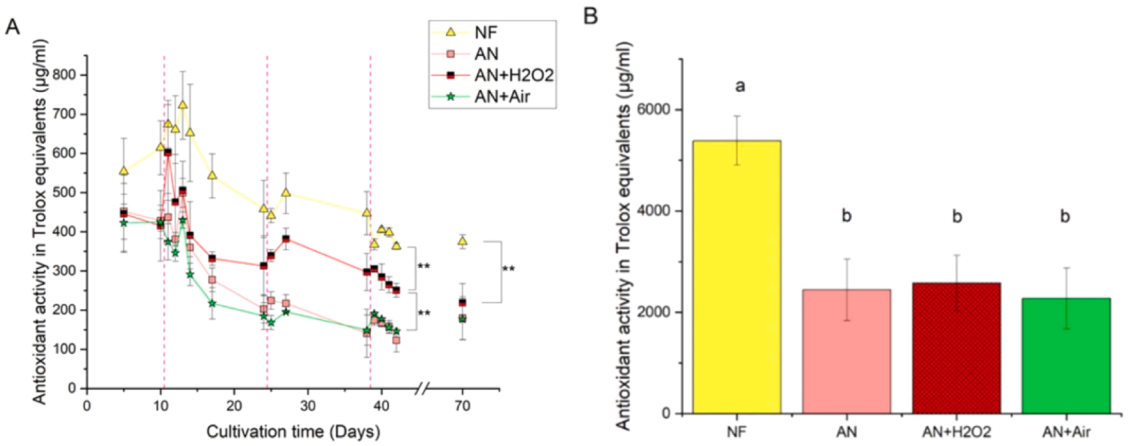

Fig. 4. Antioxidant activity of culture supernatants from the first cultivation series as Trolox equivalents (µg/ml). A) Antioxidant activity of the non-lyophilized supernatant samples directly taken from the cultures and dissolved in 70 % methanol. Dashed pink lines depict hydrogen peroxide treatment time points. Statistically significant difference between fungal culture series is depicted with brackets and asterisks, ** = $p \leq 0.01$. B) The antioxidant activity of lyophilized culture supernatants from the last time point (70 days) of cultivation. Statistically significant differences ($p < 0.01$) between the culture series are marked with different lowercase letters. NF, no fungus control flasks; AN, fermentative, anaerobic fungal culture series; AN+H2O2, fermentative fungal cultures treated with 0.1 % hydrogen peroxide; AN+Air, fermentative fungal cultures treated with air flushing.

production of mycelium under anaerobic conditions with or without H_2O_2 addition (Fig. 2F). Differences between H_2O_2 treated and non-treated cultivations incubated under same atmospheric conditions were small and statistically non-significant. This indicates that irrespective of metabolic changes observed in the cultures after addition of H_2O_2 (Fig. 2A-D), fungal growth was not suppressed by the oxidative treatment.

In contrast to the first cultivation series, laccase activity was detected in the second cultivation series only at the last time point (on day 17) in the aerobic cultures AE2 and AE2+H2O2, with activities of 1.29±0.21 nkat/ml and 1.36±0.85 nkat/ml, respectively. In the anaerobic cultures, with or without hydrogen peroxide treatment, no detectable laccase activity was observed. Therefore, it may be concluded that in the second cultivation series, the hydrogen peroxide treatment caused no inhibitory effect on laccase production or enzyme activity.

3.3. Laccase inhibition experiments

In the first cultivation series performed under fermentative (anaerobic) conditions, activity of fungal produced laccase enzyme was repressed by H_2O_2 treatment (Fig. 1A, series AN+H2O2). Due to this, we carried out experiments with a previously collected *P. radiata* high laccase activity reference enzyme by adding hydrogen peroxide and culture supernatant samples into enzyme reaction mixtures. Incubation with 0.1 % H_2O_2 caused 75 % inhibition of laccase ABTS-oxidation activity (Fig. 3A). This result indicates that hydrogen peroxide treatment to the fermentative fungal cultures may have affected negatively on activity of fungal-produced laccase enzymes in the cultures (series AN+H2O2, Fig. 1A).

However, mixing the reference enzyme with lyophilized supernatants (LS) from the 70-day no-fungus control flasks (NF) of the first cultivation series inhibited laccase activity almost completely (99 % of the enzyme activity lost, less than 1 % residual activity detected, Fig. 3A). It should be noted that this supernatant lyophilizate was from a three times hydrogen peroxide treated control flask including birch wood sawdust and yeast extract solution but no fungus. With the fungal LS samples from the same cultivation series (AN, AN+H2O2, AN+Air), inhibition of laccase enzyme was only partial resulting in 40–60 % residual laccase activity, while in the 70 % methanol solution – serving as the solvent base of the LS samples – 70 % of the original laccase activity remained (Fig. 3A).

Effect of hydrogen peroxide on formation of laccase inhibiting compounds was further studied with the LS samples of the second cultivation (Fig. 3B). With these samples, the difference between the no-fungus control (NF2) and the fungal cultures (AN2 and AE2 series) was less evident than with the extracts from the first cultivation (Fig. 3A). Also, NF2 samples from the second cultivation were less inhibitory to laccase activity (Fig. 3B) than LS samples from the first cultivation series, inhibiting approximately 70 % of the reference enzyme activity initially on day 5. Interestingly, the hydrogen peroxide treatment caused no difference in the inhibitory effect of the NF2 no-fungus control LS samples against laccase activity (Fig. 3B), leaving the origin of the high laccase inhibiting activity of the NF LS sample from the first cultivation still open for discussion. However, the inhibitory effect of the fungal aerobic culture supernatants attenuated along the course of cultivation (AE2 extracts, Fig. 3B), which indicates an ability of the fungus to eliminate or convert the inhibitory compounds (see Discussion). This further indicates that also during the first cultivation, the fungus was able to tolerate and even biotransform soluble, wood-based inhibiting compounds accumulating in the supernatants.

3.4. Antioxidant activity of culture supernatants

Initial antioxidant activities of the fungal culture supernatants from the first culture series (AN, AN+H2O2, AN+Air) on day 5 were very similar to each other (about 450 µg/ml as Trolox equivalents), with no statistically significant differences ($p > 0.05$) between the fungal culture or the non-fungal (NF) control flask samples at this timepoint (Fig. 4A). However, change in the activity occurred after day 10 following the hydrogen peroxide treatment. Addition of H_2O_2 caused a rapid increase in the antioxidant activity of AN+H2O2 series on day 11 and after the second treatment, as was observed with the NF control series (Fig. 4A). Antioxidant activities remained at markedly higher levels in the AN+H2O2 cultures when compared to the non-treated fungal (AN) or air-flushed (AN+Air) cultures on day 42 ($p < 0.01$) (Fig. 4A). Fungal culture series AN and AN+Air showed very similar antioxidant activity levels throughout the cultivation. On day 70, antioxidant activities of all fungal culture supernatants (series AN, AN+H2O2, AN+Air) were at the same level regardless of the treatment, with no statistically significant difference between the values (p values = 0.35–0.95) (Fig. 4A). This similarity was further confirmed with the final, lyophilized and pooled culture supernatant samples (Fig. 4B).

Overall, the highest antioxidant activities were measured from the NF control series supernatants (Fig. 4A), with statistically significant

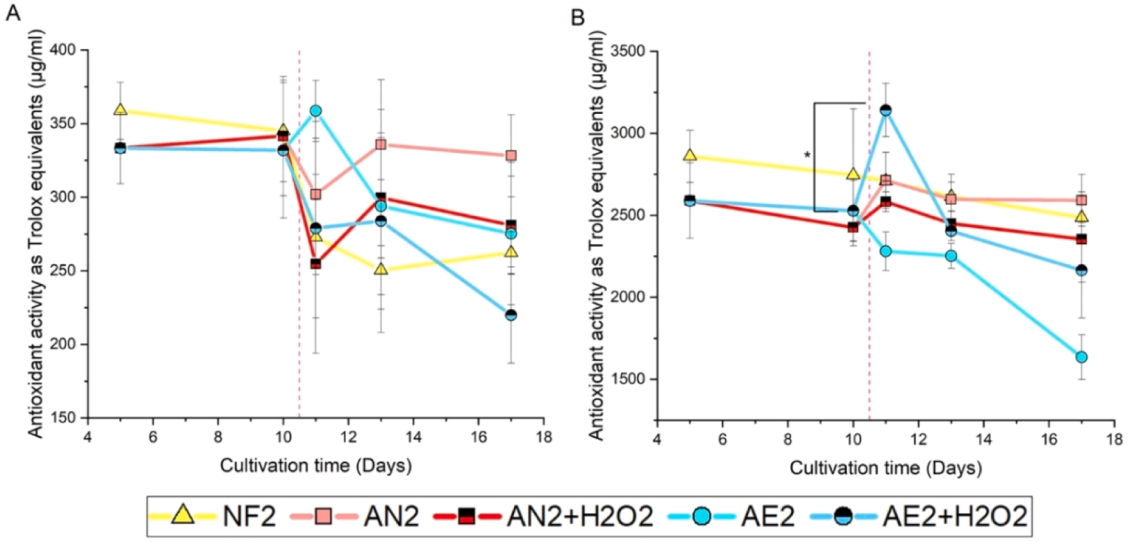

Fig. 5. Antioxidant activity of culture supernatant samples from the second cultivation series presented as Trolox equivalents (µg/ml). A) Directly in 70 % methanol dissolved culture supernatant samples, B) lyophilized supernatant samples dissolved in 70 % methanol. NF2, no fungus control; AN2, anaerobic, fermentative fungal cultures; AN2, fermentative fungal cultures treated with 0.1 % H_2O_2; AE2, aerobic fungal cultures; AE2+H2O2, aerobic fungal cultures treated with 0.1 % H_2O_2. Pink dashed line depicts the time-point for H_2O_2 treatment. Statistically significant difference ($p < 0.05$) is depicted with bracket and asterisk. The mean value and standard deviation are presented; $n = 3$ for NF2 control series samples; for fungal culture series $n = 12$ on day 5, $n = 6$ on day 10, $n = 3$ on days 11, 13 and 17.

Table 2
Antimicrobial activity of the lyophilized culture supernatants from the first cultivation series. Inhibition zone diameter (IZD) values are given in millimeters (mm). Samples showing no inhibition of microbial growth are marked as 0, and the same or lower IZD values obtained with methanol solvent are indicated in each case as n.i. = no inhibition.

Sample	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Candida albicans*	*Candida tropicalis*
NF	14	14	13	13	27
AN	0	0	0	n.i.	n.i.
AN+H_2O_2	0	0	0	n.i.	n.i.
AN+Air	0	0	0	n.i.	n.i.
Antibiotic	Kanamycin, 15	Kanamycin, 0 Penicillin G, 0	Kanamycin, 13	Fluconazole, 30	Fluconazole, 30
70 % methanol	0	0	0	8	8

differences in comparison to the fungal culture activities on cultivation days 42 and 70 (Fig. 4A,B). Interestingly, fungal growth on the wood substrate (series AN, AN+H2O2, AN+Air) apparently caused 50 % reduction in antioxidant activity of the culture supernatants at the last timepoints (Fig. 4A,B).

Effect of hydrogen peroxide treatment on antioxidant activities was further followed in the second (17-day duration) cultivation series. Directly taken fungal culture supernatant samples dissolved in methanol (Fig. 5A) showed an overall decreasing trend in antioxidant activity as was observed with the samples of the first cultivation (Fig. 4A) (except AN cultures). With the more concentrated, lyophilized supernatants from the fungal aerobic cultures, however, significant increase in antioxidant activity was observable after hydrogen peroxide treatment on day 11 (AE2+H2O2 series, Fig. 5B) ($p < 0.05$, against days 10 and 13). In all, antioxidant activities of the lyophilized culture supernatants were 8-fold in comparison to the directly in methanol diluted samples (Fig. 5A,B). This indicates that the antioxidant compounds in the culture supernatants (and released from birch wood substrate) were successfully preserved and concentrated by the lyophilization method.

3.5. Antimicrobial activity of culture supernatants

Antimicrobial disk agar-diffusion assays were performed for the 70-day lyophilized supernatants (LS, Appendix A) of the first cultivation. Non-fungal control (NF) LS samples inhibited the growth of all tested microorganisms, both the bacteria and two yeast species of *Candida,* while the fungal culture LS samples had no inhibiting effect on any of them (Table 2, Fig. 6A: plate 1, Fig. 6B: plate 1). *C. tropicalis* was the most susceptible organism to the NF supernatant showing an inhibition zone diameter (IZD) value of 27 mm, which was twice as large as for *C. albicans* and the tested bacteria (Table 2). IZD value with the antifungal reference (25 µg fluconazole/disk) was 33 mm for *C. albicans* and 30 mm for *C. tropicalis*, which was within the accepted quality control range of inhibition (26–37 mm) (NCCLS, 2004). Susceptibility of *C. tropicalis* to the no-fungus NF LS sample was exceptional and may thereby indicate presence of wood substrate and medium-derived soluble compounds demonstrating specific antifungal effect. It is noteworthy that the 5 % yeast extract solution, either treated or non-treated with HCl, other acids or NaOH, caused large IZD zones for *C. tropicalis* (Fig. 6B: plates 7–9). This indicates that most of the antifungal activity in the no-fungus NF LS samples (Fig. 6B: plates 1,2) may be due to presence of yeast extract in the substrate solution.

For the Gram-positive bacterium *S. aureus,* IZD of the NF sample was 14 mm, which was close to the inhibition caused by the reference antibiotic (kanamycin, 5 µg/disk). With the Gram-negative bacterium *E. coli* the effect of the NF sample was also equal to the kanamycin control IZD (13 mm). Interestingly, although *P. aeruginosa* was not inhibited by either of the reference antibiotics (kanamycin and penicillin), it was inhibited by the NF LS sample (IZD 14 mm). As observed with species of *Candida*, LS samples from fungal cultures of the first cultivation series caused no effect on the tested bacteria (Table 2). The 70-day LS samples were also tested after catalase treatment (Appendix A) against *C. tropicalis*. Interestingly, catalase-treated NF samples had no inhibiting activity whereas lyophilized and methanol reconstituted non-

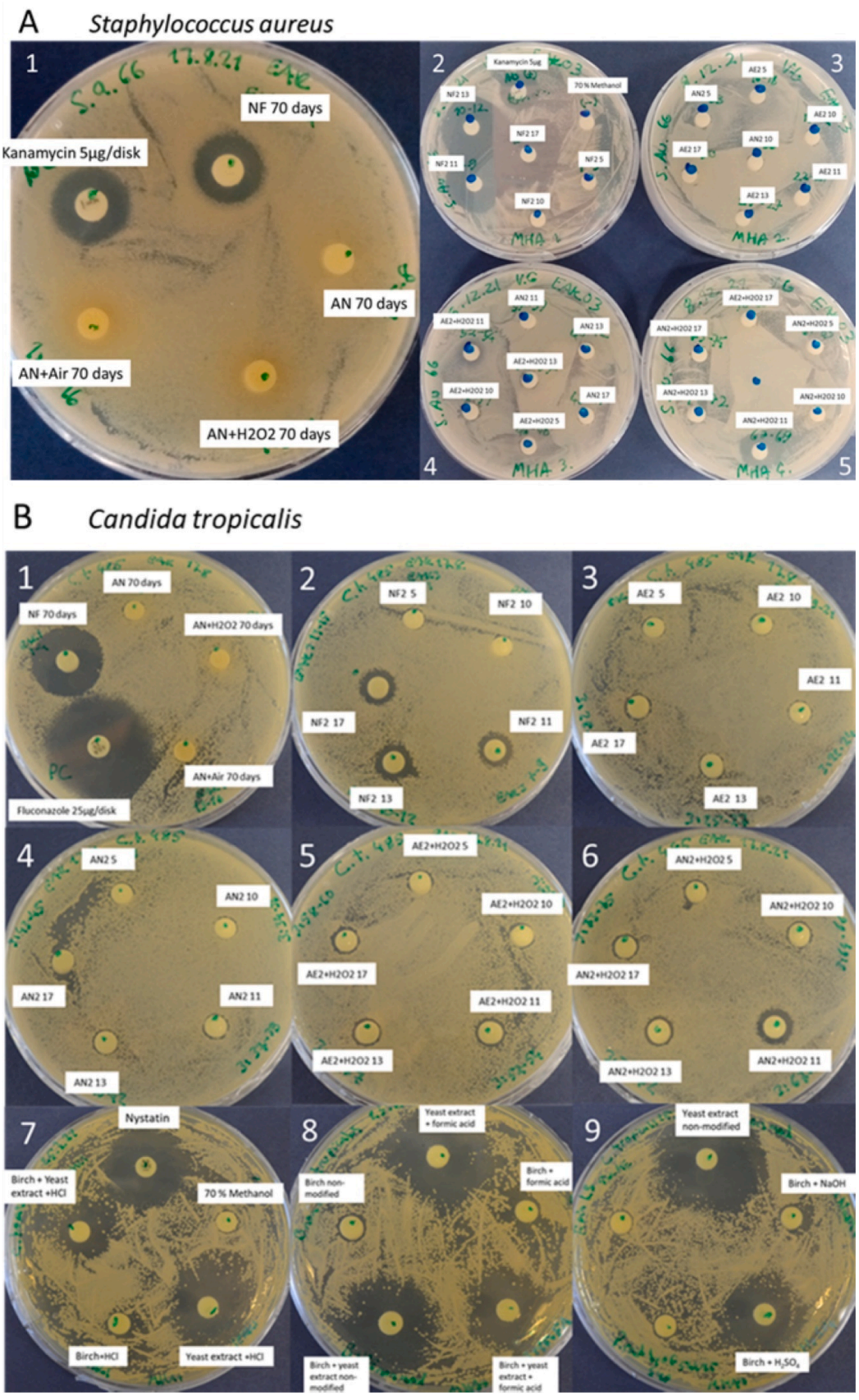

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Fig. 6. Inhibition of growth of A) *Staphylococcus aureus* and B) *Candida tropicalis* by lyophilized supernatant (LS) samples from *P. radiata* cultures on birch wood. On figures A and B: plates 1 comprise tested samples from the first cultivation (day 70 end samples), while plates 2–6 comprise LS samples from the second cultivation (days 5 to 17). Plates 7–9 comprise substrate control and solvent (70 % methanol in water) test samples. For *S. aureus*, kanamycin (A, plate 1), whereas for *C. tropicalis*, fluconazole (B, plate 1) and nystatin (B, plate 7), were adopted as positive reference compounds. NF, NF2, no fungus control flask supernatants; AN, AN2, fermentative anaerobic fungal cultures; AE2, aerobic fungal cultures; +H2O2, hydrogen peroxide treatment; +Air, air flushing treatment. Birch, birch sawdust wood + water; yeast extract, autoclaved 5 % yeast extract solution in water; +HCl, +H_2SO_4, +formic acid, adjustment to pH 3 in the solution by addition of respective acid.

Table 3
Antimicrobial activity of lyophilized culture supernatants from the second cultivation. Inhibition zone diameter (IZD) values are given in millimeters (mm). Zero IZD values or values below the 70 % (v/v) methanol solvent control generated IZD are marked with 0. Cultivation day samples 5 and 10 and cultivation series AE2 and AN2, in which no inhibition was found were not included in the table. NF2, no fungus control flasks; AN2+H2O2, fermentative fungal cultures treated with hydrogen peroxide; AE2+H2O2, aerobic fungal cultures treated with hydrogen peroxide.

Tested microbe and sample	Solvent (70 % methanol)	Reference antibiotic	Culture supernatant sample taken on		
Staphylococcus aureus	0	Kanamycin, 16	*Day 11*	*Day 13*	*Day 17*
NF2			24	26	25
AE2+H2O2			11	0	0
AN2+H2O2			16	0	0
Candida tropicalis	8	Fluconazole, 30	*Day 11*	*Day 13*	*Day 17*
NF2			10	10	10
AE2+H2O2			8	0	0
AN2+H2O2			10	8	0

catalase treated 0.3 % H_2O_2 sample produced an IZD of 13 mm suggesting that some hydrogen peroxide may have been present in the samples even after lyophilisation. The lyophilized catalase-treated 0.3 % H_2O_2 sample, however, caused no inhibition of growth.

With culture supernatants from the second cultivation, a surge in antimicrobial activity was observed with no-fungus control (NF2) LS samples taken after hydrogen peroxide treatment on day 11 and further on. NF2 LS samples strongly inhibited the growth of *S. aureus* (IZD values 24–26 mm) and *C. tropicalis* (IZD values 10 mm) (Table 3, Fig. 6A: Plate 2, Fig. 6B: Plate 2). Regarding fungal cultures, antimicrobial activities were observed after hydrogen peroxide treatment under both atmospheric conditions in LS samples from day 11 (series AN2+H2O2 and AE2+H2O2), however, attenuating and disappearing after this timepoint (Table 3).

3.6. Antifungal effect of the culture supernatants against P. radiata itself

Lyophilized supernatants (LS) from the first cultivation were also tested against *P. radiata* itself to find out, if the fungus could be susceptible to similar growth-inhibiting effect observed with the yeast-like ascomycete *C. tropicalis* (Table 2, Table 3, Fig. 6B). With the NF (no-fungus control) LS sample, similar antifungal effect was observed against *P. radiata* mycelium cultivated on malt extract agar (MEA) medium (IZD 45±8 mm, Plate 1, Fig. 7). Interestingly, antifungal effect of NF LS disappeared with catalase treatment (Plate 3, Fig. 7). However, no inhibition of growth was observed with lyophilized 0.3 % H_2O_2 solution or the 70 % methanol solvent control (Plate 3, Fig. 7). It is obvious that *C. tropicalis* was far more susceptible against H_2O_2 (and methanol solvent) (Fig. 6B) than *P. radiata*. Disappearance of fungal growth inhibiting activity in the NF LS due to catalase treatment (Plate 3, Fig. 7), however, remains puzzling. The fungal culture LS samples, on the contrary, showed no inhibiting activity against *P. radiata* even when applied in higher dose (37 μl) than the NF LS samples (plate 4, Fig. 7). Notable is that of the two antifungal references only the nystatin polyene compound was effective (IZD 25±4 mm, Plates 1–5; IZD 31 mm, Plate 6; Fig. 7) whereas *P. radiata* showed complete resistance towards fluconazole (Plate 6, Fig. 7). Resistance to fluconazole contrasts to what was observed with the two, both fluconazole-sensitive species of *Candida* (Table 2, Table 3, Fig. 6B).

3.7. LC-MS analysis of aromatic compounds

In the no-fungus control flask LS samples, which showed high antimicrobial activities (series NF2, Fig. 6A,B), several UHPLC-UV-DAD chromatogram peaks of aromatic compounds appeared disappeared on days 10 and 11 of cultivation following hydrogen peroxide treatment (Fig. 8, Appendix B). According to mass spectral analysis, several of the emerging compounds were chlorinated, as revealed by their isotope patterns (Appendices B, C). Isotope patterns of chlorination at the organic compounds were also detected in positive ionization mode indicating that chlorine atoms were covalently attached and not present as ionic adducts.

As an example of the chlorinated compounds detected in the NF2 supernatants, a peak eluting at 4.02 min (UV, 525 *M/z* es-) disappeared after treatment with H_2O_2 whereas another peak eluting at 5.52 min (UV, 559 *M/z* es-) appeared and was detected until the end of cultivation (day 17) (Fig. 8). The two compounds presented similar UV spectra (extinction maxima near 280 nm) with a mass difference of 33.9597 Da, equaling to loss of one proton and gain of one chlorine atom. The compound eluting at 4.02 min was putatively identified as [3,4,5-trihydroxy-6-(3,4,5-trimethoxyphenoxy)oxan-2-yl]methyl-4-hydroxy-3,5-dimethoxybenzoate by its mass fragmentation pattern (Appendix C). The compound is a known natural product in downy birch (European white birch, *Betula pubescens*) inner bark (Pan and Lundgren, 1994) (see Discussion). Similarly, the peak eluting at 5.52 min was putatively identified as its chlorinated derivative possessing one chlorine attached covalently to one of the two unsubstituted carbons of the 3,4,5-trimethoxyphenoxy- moiety of the birch compound (Appendix C). The chlorinated compound peak was among the largest appearing after H_2O_2 addition (Appendix B). The same compound – albeit in somewhat smaller peaks - appeared also in the AE2+H2O2 and AN2+H2O2 fungal culture supernatants after H_2O_2 treatment but not in the non-treated fungal cultures (series AE2 and AN2) (Appendix B). The chlorinated compound peak, however, diminished and disappeared in the H_2O_2 treated fungal cultures (not found any more on day 17) while the non-chlorinated compound peak started to increase in size.

4. Discussion

As predicted, the fungus produced more mycelium on birch wood substrate under aerobic conditions than in the fermentative, anaerobic cultures (results from the second cultivation series), similar to our previous observations on spruce wood and lignocellulose waste material substrates (Mattila et al., 2017, 2020). Higher production of mycelial biomass by *P. radiata* correlated with more rapid utilization of sugars released and accumulating from the birch wood cultures, likewise noticed previously (Mattila et al., 2017). In our current study, cultivation atmosphere (aerobic *versus* fermentative anaerobic) and oxygen availability affected laccase production by *P. radiata*. The differences between fermentative (AN and AN+H2O2, AN2 and AN2+H2O2) and the aerobically incubated (AE2, AE2+H2O2, AN+Air) fungal cultures in laccase activities was obvious in both cultivation series performed on the birch wood substrate.

Production of laccase and manganese peroxidase (MnP) by *P. radiata* fungus fluctuate temporally in long-duration cultures on lignocellulose

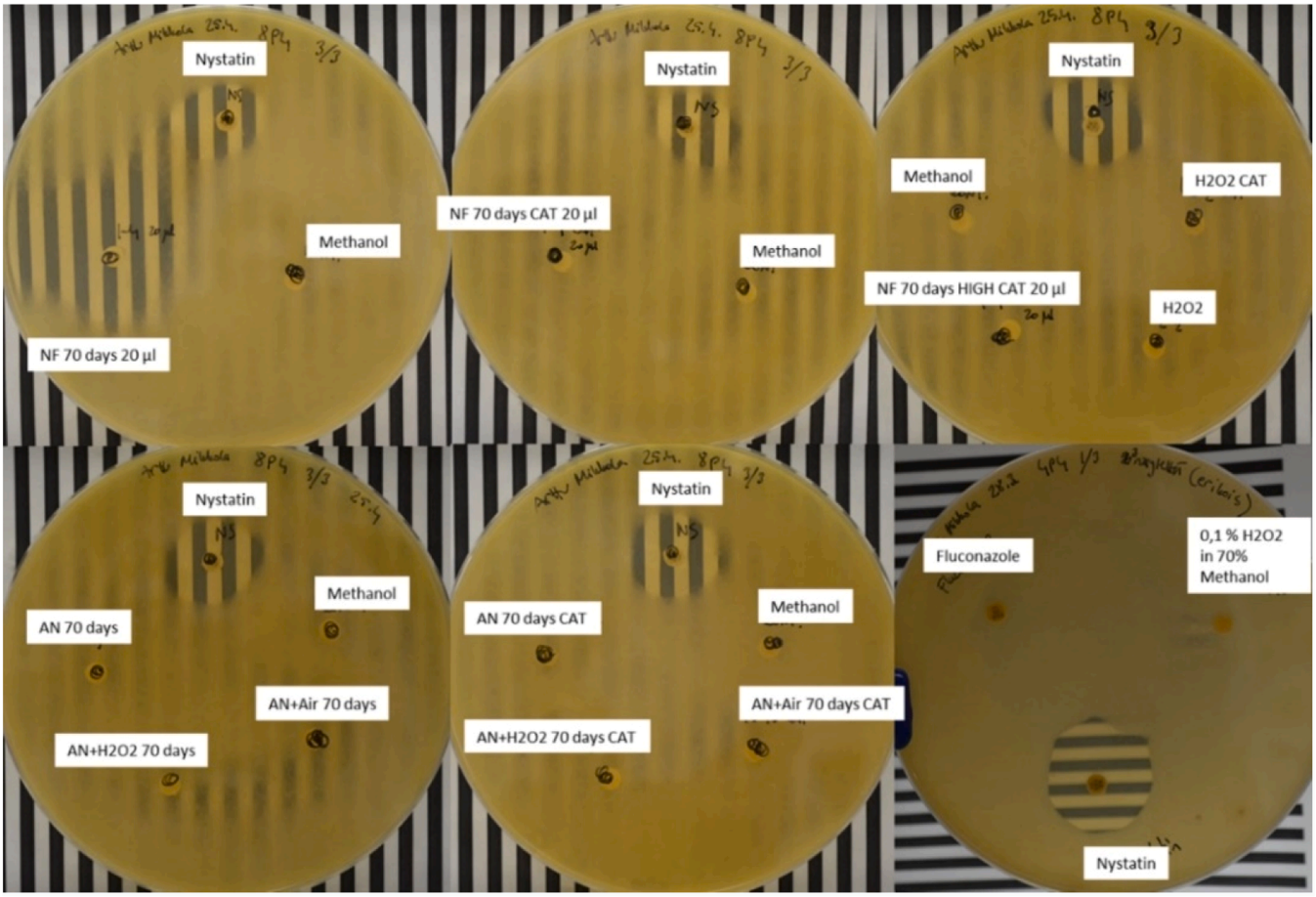

Fig. 7. Antifungal activity of the lyophilized culture supernatant samples from the first cultivation series against *Phlebia radiata*. NF, no fungus control flasks; AN, fermentative (anaerobic) fungal culture series; AN+H2O2, fermentative fungal cultures treated with 0.1 % hydrogen peroxide; AN+Air, fermentative fungal cultures treated with air flushing. CAT, catalase treated samples. NF samples with or without catalase treatment were applied as 20 µl/ 2 stacked blank antimicrobial test disks whereas other samples were applied as 37 µl/ 2 stacked blank antimicrobial test disks.

substrates (Mali et al., 2017; Veloz Villavicencio et al. 2020). Typically, *P. radiata* laccase production starts early on, at least during the first nine days of cultivation depending on the medium and substrates, supplements, cultivation conditions and culture scale (Mäkelä et al., 2006; Kuuskeri et al., 2015; Mali et al., 2017; Veloz Villavicencio et al., 2020). Noticeably, absence of laccase and MnP activities under anaerobic cultivation conditions (Mattila et al., 2020) and later detection of laccase and MnP activities after two weeks of cultivation under "semi--aerobic conditions" (Mäkinen et al., 2018) were observed in our previous studies.

A major difference between cultivation series in this study was in the initial adjustment to pH 3 made in the culture flasks' water phase in the second cultivation series, which may have postponed onset of laccase production. Adjustment to low pH was done in the cultures to promote softening and degradation of the wood sawdust substrate, thereby potentially allowing faster mycelial growth and metabolic activity after inoculation. However, increased acidity in the beginning of the cultivation apparently prevented fungal metabolism and enzyme production, as was observed with inhibition of ethanol production and laccase activity in this study. In a study with *Phlebia floridensis*, higher laccase and MnP activities were produced at pH values 4 and 5, while at more acidic conditions, at pH values 3 and 2, enzyme production was markedly lower or even zero, respectively (Arora and Rampal, 2002). A previous experiment with *P. radiata* at pH 3 showed missing laccase activity at fermentative anaerobic conditions (Mattila et al., 2020) in agreement to our results, while at pH >4.5 laccase has been produced by the fungus, also earlier than in our study (Mäkelä et al., 2006; Kuuskeri et al., 2015, 2016; Mali et al., 2017; Mäkinen et al., 2018; Veloz Villavicencio et al., 2020). These findings point to the significance of three factors: culture atmosphere, lignocellulose substrate and initial pH of the medium on white rot fungal metabolism and enzyme production.

Hydrogen peroxide treatment made a negative impact on *P. radiata* laccase activities as well as production of ethanol under fermentative (anaerobic) conditions. Laccase activity disappeared from the culture supernatants after H_2O_2 addition (in the first cultivation series), and the harmful effect was demonstrated in laccase inhibition experiments. Under fermentative (anaerobic) conditions, fungal production of ethanol was likewise disturbed by hydrogen peroxide treatment, leading to disappearance of both ethanol and acetate (acetic acid) for a few days. Under oxygen-depleted conditions on lignocellulose substrate, the fungus produces ethanol and some acetic acid through up-regulation of alcohol fermentation and phosphoketolase pathways (Mattila et al., 2020). In water solution including ferric salts, hydrogen peroxide may oxidize ethanol first to acetic acid and finally, to carbon dioxide and water (Walton and Christensen, 1926). This may explain, why both ethanol and acetate disappeared so rapidly in the cultures after addition of H_2O_2. However, both laccase activity (under aerobic conditions) and ethanol production (under anaerobic fermentative conditions) were restored within a few days, which demonstrates resilience and metabolic recovery of the fungal mycelium quickly after harsh oxidative stress (Fig. 9).

The mechanism of disappearance of laccase activity may be explained by oxidative damage or denaturation of laccase enzyme proteins by the high dosage of H_2O_2 in the solution. Hydrogen peroxide can

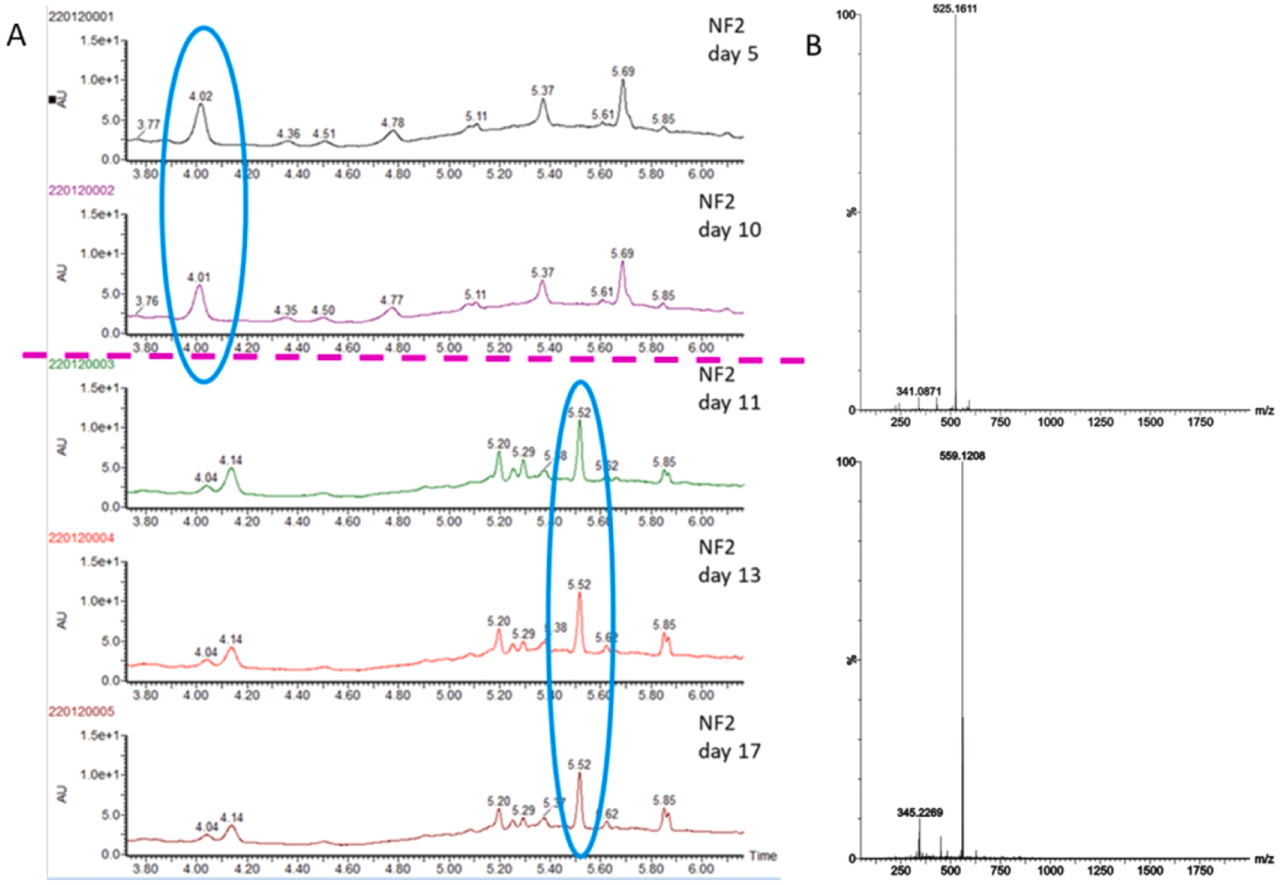

Fig. 8. Emerging and disappearing peaks of aromatic compounds in the UHPLC-UV-DAD chromatograms of supernatant samples from no-fungus control (NF2) flasks of the second cultivation. A) Chromatograms before (days 5 and 10) and after (days 11, 13 and 17) addition of hydrogen peroxide (horizontal dashed pink line depicts the timepoint). Blue circles highlight the birch compound peak at retention time of 4.02 min and the appearing chlorinated compound peak at 5.52 min. B) ES-Mass spectra of the RT 4.02 min disappearing peak (above) and the RT 5.52 min appearing peak (below). Mass difference between the compound ion peaks was 33.9597 Da (*m*/*z*).

affect protein function and enzyme activity loss by oxidation as was recently shown with secreted proteins of the white rot fungus *Trametes versicolor* (Castaño et al., 2021). With other fungi subjected to H_2O_2 treatment, highly dose and time-dependent results have been obtained (Wiberth et al., 2019; Hu et al., 2020). Alternatively, inhibiting compounds may have emerged into the culture supernatant by leaching from the birch wood substrate and/or by modifications like H_2O_2-caused oxidation and chlorination of the wood-dissolved compounds (Valette et al., 2017; Belt et al., 2018). Modification of culture supernatant compounds by halogenation was detected in the second cultivation series. Accumulation of wood-derived inhibitory compounds in the cultures after H_2O_2 treatment was further supported by laccase inhibition experiments and bioactivity tests of the culture supernatants.

Mechanism of laccase activity inhibition could be due to the wood-derived compounds reacting with the assays' first reaction product ABTS radical, resulting with charge transfer mediation and delayed production of the radical product – the property in use in laccase-mediator oxidation systems (Bourbonnais et al., 1998). It is likely that the antioxidant-active compounds in the culture supernatants have disturbed laccase assay measurements by reduction of the ABTS radicals. ABTS is commonly applied in food antioxidant tests where the generated ABTS radical cation is reduced back by antioxidant compounds (*Re* et al., 1999; Huang et al., 2005). Notably the 0.1 % (w/v) hydrogen peroxide treatment suppressed only 70 % of the initial laccase activity in the ABTS assay, while addition of the NF LS sample suppressed laccase activity by more than 99 % in comparison to the solvent (methanol) control alone. This level of inhibition (accumulation of inhibiting compounds) in the NF supernatant would be likely to suppress laccase enzyme function. It is possible that no laccase activity could be observed before the fungus was able biotransform and eliminate these compounds.

Hydrogen peroxide treatments apparently boosted antioxidant activity in the fungal culture supernatants extracted from the birch-wood substrate cultivations. This could be seen in rapid rise of antioxidant activity in the culture samples one day after addition of H_2O_2. In the course of cultivation, however, a decreasing trend in antioxidant activities was observed. This is well in line with our observation that both the number of compound peaks in the UPLC chromatograms as well as peak areas were reduced in the fungal cultures when compared to the non-fungal controls. Results of antioxidant activity assay and laccase inhibition experiment correlated with each other indicating that with higher antioxidant potential of the culture supernatant, the inhibition of laccase activity was more severe. This furthermore points to the release and accumulation of redox active compounds such as wood phenolics into the water phase in the fungal cultures. Birch (*Betula* spp.) wood is rich in phenolics and syringyl-type lignin moieties (Sjöström, 1981; Saka and Goring, 1988), and phenolic compounds are common reducing substrates for laccase enzymes (Hildén et al., 2009; Lundell et al., 2010; Agustin et al., 2021). Antioxidant properties of lignin can even be enhanced by laccase enzymatic activity (Li et al., 2018). However, since the antioxidant activity effect as well as inhibition of laccase activity caused by supernatants taken from the NF2 no-fungus control flasks were not affected by hydrogen peroxide treatment, while antimicrobial activities of these samples were substantially changed by H_2O_2 addition,

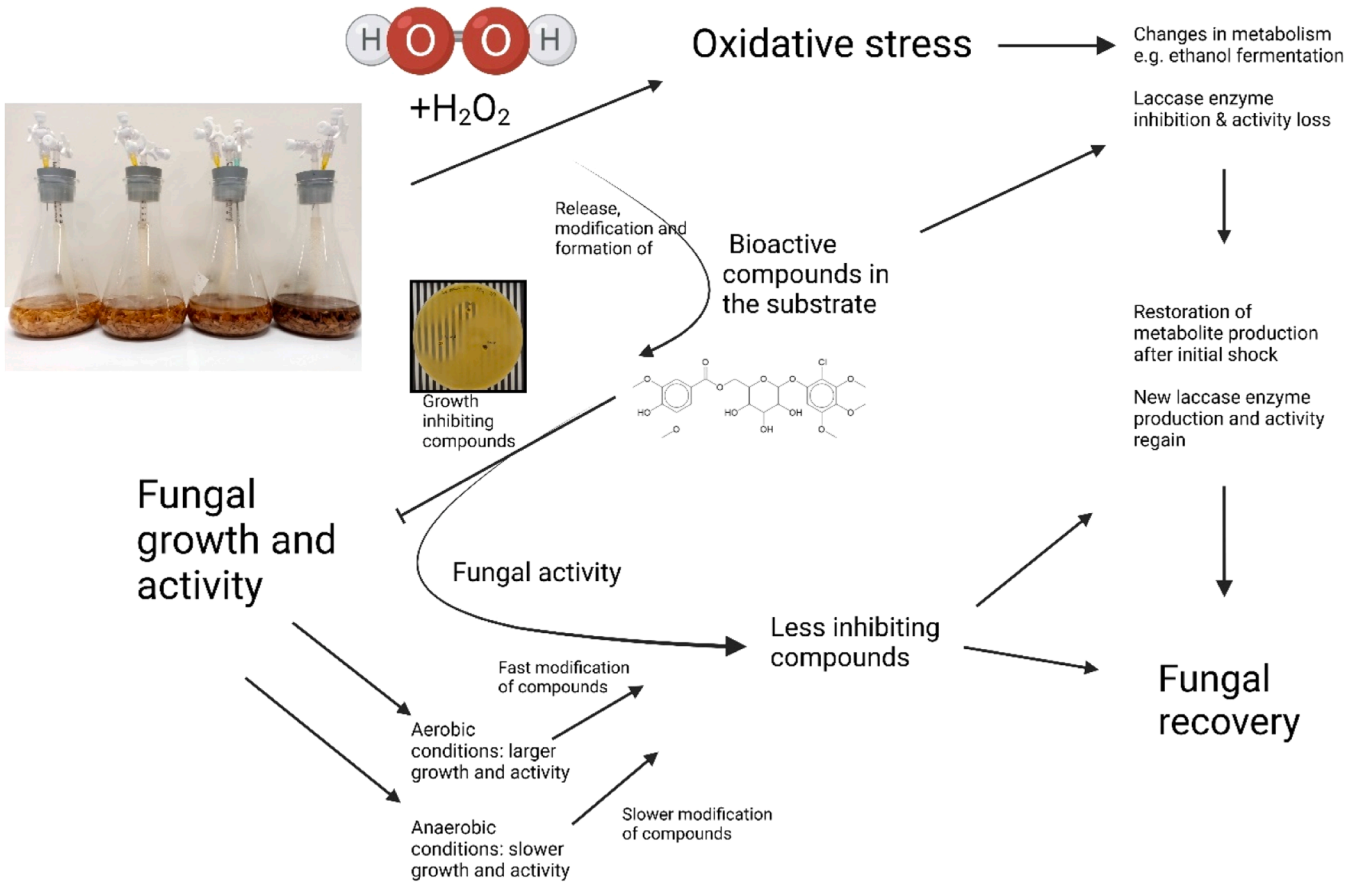

Fig. 9. Response to oxidative stress effected by H_2O_2 in white rot fungal cultures on birch wood. Putative scheme was created with BioRender.com according to results obtained in this study.

the soluble compounds responsible for the variant bioactivities apparently are not the same. Regarding antimicrobial activities, synergistic effects of chemically variant compounds cannot be ruled out.

The birch-wood culture supernatants also presented antimicrobial activity towards several bacterial and yeast species. The lyophilized no-fungus samples had an inhibiting effect towards test microbes representing different taxa. This suggests presence of a set of wood-substrate and medium-yeast extract derived compounds with variant modes of action against the target organisms. Inhibition of growth of the Gram-positive bacterium *Staphylococcus aureus* and the human-pathogenic yeast fungus *Candida tropicalis* was established by the no-fungus NF substrate lyophilized extracts, and hydrogen-peroxide treated fungal birch-wood culture supernatants. No clear inhibition was visible with the culture supernatant samples before the treatment, but one day after H_2O_2 addition, the antimicrobial effect was apparent for both tested microbes.

Effect of H_2O_2 treatment on accumulation and formation of potential antimicrobial and antifungal compounds was observed especially in the second cultivation series, with the strongest effect caused by no-fungus NF2 samples one day after H_2O_2 addition. This indicates that the effective compounds were leached from the birch wood and yeast extract substrates, and modified by addition of H_2O_2. By LC-MS analysis, we found that multiple organic compounds in these extracts were chlorinated, which explains the strong antimicrobial activity of the supernatants. Many antibacterial compounds are chlorinated or brominated (halogenated) (Smyrniotopoulos et al., 2010; Konečná et al., 2022; Tenebro et al., 2023) and fungi can produce chlorinated organic compounds as secondary metabolites (Öberg et al., 1997; Duan et al., 2017; Niu et al., 2021). It is possible that addition of hydrogen peroxide into the cultures containing some hydrochloric acid (which was used for adjustment of pH in the second cultivation) may have oxidized and chlorinated the soluble organic compounds of birch wood. Our finding of a chlorinated derivative of the natural birch wood (inner bark) compound 3,4,5-trimethoxyphenyl-6-O-syringoyl-β-d-glucopyranoside ([3,4,5-trihydroxy-6-(3,4,5-trimethoxyphenoxy)oxan-2-yl]methyl-4-hydroxy-3,5-dimethoxybenzoate) (Pan and Lundgren, 1994) furthermore supports this reaction pattern.

Observed antimicrobial activities were well in line with emergence and disappearance of chlorinated compounds in the birch-wood fungal cultures. Furthermore, ability of the fungus to eliminate antimicrobial activity of the supernatants, while re-converting the modified chlorinated compounds to their original form, was obvious. In fungal cultures, some of these compounds may have biological origin. Chlorine is a ubiquitous element present in the environment, also in wood, and production of organochlorine compounds during wood degradation by white rot fungi has been shown previously (Öberg et al., 1997).

The no-fungus control flask (NF) lyophilized samples prevented hyphal growth of *P. radiata* in the agar medium plate assay. However, catalase-treated NF sample had no such growth-inhibiting effect, indicating that the supernatant may have included residual hydrogen peroxide. Insufficient removal of H_2O_2 from the samples during lyophilization was thereby considered as one explanation for inhibitory action of the samples towards microbes and laccase enzyme. On the other hand, H_2O_2 treatment of NF2 supernatants caused no additional inhibiting effect on laccase activity whereas antimicrobial activity increased substantially. However, it is questionable that residual hydrogen peroxide could be the antifungal agent since lyophilized (0.3 % hydrogen peroxide) or non-lyophilized (0.1 % hydrogen peroxide) solution caused no prevention of hyphal growth of *P. radiata*. Regarding this, the observed loss of growth-prevention in NF2 samples after catalase

treatment must then be explained by other mechanisms than elimination of hydrogen peroxide.

Taking into account results from antimicrobial assays and compound profiles of UPLC analyses it seems likely that the fungus converted or partially eliminated the harmful soluble compounds emerging into the birch wood substrate cultures (Fig. 9). Hydrogen peroxide, other reactive oxygen species and various radicals are important agents in the fungal extracellular degradative processes against wood and lignocelluloses (Hammel et al., 2002; Lundell et al., 2014; Bissaro et al., 2018; Castaño et al., 2018; Mattila et al., 2022). Generation and utilization of reactive oxygen species is essential not only for wood decay fungi but in general, for soil-inhabiting as well as plant and animal pathogenic species. Filamentous fungi have effective enzymatic and biochemical machinery to eliminate reactive and harmful compounds (Mattila et al., 2022) which apparently is essential in competition of living space and nutrients in their demanding habitats.

5. Conclusions

Although the oxidative stress caused by addition of hydrogen peroxide affected fungal metabolism (as was seen in temporary drop in fermentation product concentrations) and laccase enzyme activity, the fungus was able to restore its growth and activities after the treatments. Addition of hydrogen peroxide caused a transient negative effect on fungal functions with concurrent release of bioactive compounds derived from the substrate. However, fungal cultures recovered quickly from the oxidative effect and eliminated the harmful and chlorinated compounds (Fig. 9). Recovery of the fungus, as well as elimination of the birch-derived aromatic and chlorinated compounds was more rapid under aerobic than fermentative (anaerobic) conditions. Which are the molecular mechanisms against oxidative stress and how *P. radiata* experiences hydrogen peroxide treatment at transcriptome (gene expression and regulation) level, these are our next interests. Versatile effects of hydrogen peroxide on the fungal cultures like transient suppression of laccase enzyme activity and emergence of interfering and antimicrobial natural compounds, all these observations deserve further attention.

Appendices. Appendix A, Additional methods. **Appendix B**, LC-MS data of aromatic compounds in the culture supernatants. **Appendix C**, Fragmentation pattern of the identified compounds.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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