Agar Plate-Based Screening Approach for the Identification of Enzyme-Catalyzed Oxidations

Alina Kinner, Stephan Lütz, and Katrin Rosenthal*

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Biocatalytic oxidation reactions are in high demand. Among the applied enzymes, the heme-thiolate enzyme subfamily of unspecific peroxygenases (UPOs), which relies on hydrogen peroxide as cosubstrate and oxidant, has generated great interest. Almost two decades after their first description, databases provide thousands of putative UPO sequences, but only a few enzymes have been characterized. To address this gap, efficient screening methods for the identification of novel UPOs are necessary. Here, a new screening strategy is presented based on solid cultivation of wild-type fungi. The identification of a promising candidate strain highlights the applicability of this approach.

Keywords: ABTS, Activity screening, Laccase, Unspecific peroxygenase, Wild-type fungi

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1 Introduction

The selective oxyfunctionalization of organic molecules is one of the most challenging tasks in synthetic chemistry. The enzyme class of oxidoreductases holds great potential for industrial and pharmaceutical applications with its members such as cytochrome P450 monooxygenases (P450s), laccases or unspecific peroxygenases (UPOs) [1]. For example, there is considerable interest in using laccases for dye degradation and synthesis of aromatic compounds [2]. The versatile group of P450s is a widely used biocatalyst for C-H functionalization [3]. In addition, the exclusively fungal family of robust C-H-oxidizing UPOs has also turned out to be a promising alternative for the oxyfunctionalization of organic substrates. In 2004, the heme enzymes were first described in the basidiomycete *Agrocybe aegerita* (*Aae*UPO) [4]. Almost two decades later, databases provide up to 4800 putative UPO sequences derived from fungi throughout the fungal kingdom, but only 18 enzymes have been purified and characterized in detail [5, 6]. In contrast to chloroperoxidases (CPOs), UPOs poorly chlorinate but otherwise efficiently catalyze a broad range of selective oxyfunctionalization of non-activated C-H- and C=C-bonds as well as C-C-bond cleavage. Comparable to the peroxygenase mode of some P450s, the heme-thiolate enzymes catalyze a broad range of H_2O_2-dependent oxidations without the need for NAD(P)H or electron transfer proteins and can thus be considered as self-sufficient [7]. In the resting state of the enzyme, water is loosely bound to the ferric heme, then replaced by H_2O_2 to form the peroxo-complex Compound 0 and subsequently the key intermediate Compound I, an oxo-ferryl cation radical complex. Compound I, a strong oxidant, abstracts a hydrogen atom from the substrate, which forms the protonated intermediate Compound II. Finally, the hydroxylated product is released after rapid recombination of the short-lived substrate radical. From a catalytic perspective, UPOs are assumed to be the missing link between P450s and CPOs, as they can perform one-electron and two-electron oxidations [8].

Generally, all UPOs contain highly conserved amino acid motifs essential for their catalytic functionality. On the other hand, there are several structural differences in UPO architecture, such as the heme access channel, which might explain the differences in substrate specificity [9]. For instance, neither *Aae*UPO nor *Mro*UPO from *Marasmius rotula* convert the sterically demanding compound testosterone, while *Cgl*UPO from *Chaetomium globosum* is able to oxidize the steroid molecule [10]. Several patents on UPO-sequences as well as UPO-catalyzed reactions indicate their potential for industrial application covering the hydroxylation of aliphatic hydrocarbons to deacylation of corticoid [11–18].

Alina Kinner, Prof. Dr. Stephan Lütz, Dr. Katrin Rosenthal
katrin.rosenthal@tu-dortmund.de
TU Dortmund University, Chair for Bioprocess Engineering, Department of Biochemical and Chemical Engineering, Emil-Figge-Straße 66, 44227 Dortmund, Germany.

As a result of their catalytic properties, they have been the subject of recent investigations ranging from genome mining and enzyme activity screening to protein and reaction engineering [19–21]. Since UPO secretion often only occurs after one to three weeks of fungal cultivation during secondary metabolism, published screening approaches for new wild-type enzymes were mainly performed using heterologous expression rather than the original fungus [5]. Practical and faster screening methods are needed to investigate the natural resources of UPO proteins and to exploit the potential of wild-type strains.

In this work, a new screening approach is presented focusing on solid cultivation of fungi in combination with enzyme activity assays at microliter scale. The applicability of this approach was underscored by the identification of a new fungus as promising candidate for UPO expression.

2 Experimental

2.1 Organisms

Agrocybe aegerita DSM 22459, *Bipolaris victoriae* DSM 62621, *Bipolaris zeicola* CBS 237.77, *Caldariomyces fumago* DSM 1256, *Coprinus radians* DSM 888, *Pyrenophora tritici-repentis* CBS 265.80, *Neofusicoccum parvum* CBS 133503, *Talaromyces stipitatus* CBS 375.48 were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany) and the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands).

2.2 Culture Conditions

Fungal pre-cultures were inoculated from glycerol stocks and grown on malt extract agar (ROTH, Germany) at 24 °C for two weeks. For liquid main cultures, 250-mL shake flasks containing 50 mL liquid medium were inoculated with a half-overgrown pre-culture plate and cultivated at 24 °C and 100 rpm for four weeks. Soybean meal-based medium (SG) consisted of soybean meal (30 g L^{-1}) (Hensel Voll-Soja; Walther Schoenenberger Pflanzensaftwerk GmbH & Co. KG, Germany) and glucose (40 g L^{-1}). Soy peptone-based medium (GYP) contained soy peptone (48 g L^{-1}), glucose (42 g L^{-1}) and yeast extract (4.5 g L^{-1}).

For agar plate main cultures, a 5×5 mm overgrown agar slice, which corresponded approximately to a volume of 75 mm^3, was cut and transferred to a fresh agar plate containing soybean meal (30 g L^{-1}) and agar-agar (20 g L^{-1}). Plates were incubated at 24 °C for three weeks.

2.3 Enzyme Activity Assays

During fungal main cultivation, samples were taken weekly and enzyme activities were monitored. During liquid cultivation, 1 mL of culture broth was sampled, centrifuged to remove the fungal mycelium, and the supernatant was stored at –20 °C until its use for the activity assays. In case of agar plate cultivation, a 5×5 mm overgrown agar slice was cut and transferred into a 2-mL reaction tube.

2.3.1 NBD Assay

For liquid cultivation, the screening for UPO activity was performed in 200 µL assay mixture in 96-well microtiter plates (No. 655101, Greiner Bio-One™, Austria). The reaction mixture contained 100 µL 100 mM potassium phosphate buffer (pH 7.0), 40 µL 5 mM 5-nitro-1,3-benzodioxole (NBD; in 100 % acetonitrile) and 40 µL culture supernatant. The reaction was started by adding 20 µL 10 mM H_2O_2 and conducted at room temperature for 20 min. NBD oxidation was followed by the formation of yellow-colored 4-nitrocatechol (4-NC), which was verified by a characteristic color shift from yellow (absorbance at 425 nm) to red at pH ≥12 after addition of 15 µL 5 M NaOH (final absorbance at 514 nm) [22]. After 20 min, absorbance was measured before and after addition of NaOH using the FLUOstar® Omega microplate reader (BMG Labtech, Germany).

For the agar slice assay, the reaction mixture contained 500 µL 100 mM potassium phosphate buffer (pH 7.0), 200 µL 5 mM NBD, 200 µL ddH_2O, 100 µL 10 mM H_2O_2 and one agar slice in a 2-mL reaction tube. The tubes were incubated for 1 h at room temperature and 600 rpm. Afterwards, 200 µL of reaction mixture were transferred into a 96-well microtiter plate for absorbance measurements at 425 nm in the microplate reader. Then, 15 µL 5 M NaOH were added and absorbance at 514 nm was measured. The concentration of formed 4-NC was calculated by a standard dilution series (8–250 µM). To exclude any side reactions of the medium, equal amounts of sterile SG or GYP medium as well as a sterile soybean meal agar slice were used as controls instead of fungal samples. The respective medium control served as blank. For spectrum scans, the buffer was used as blank.

2.3.2 ABTS Assay

For liquid cultivation, the screening for UPO and laccase activities was performed in 200 µL assay mixture in 96-well microtiter plates (No. 655101, Greiner Bio-One™, Austria). The reaction mixture contained 100 µL 50 mM sodium citrate buffer (pH 4.5), 40 µL 1.2 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 40 µL culture supernatant. The reaction was started by adding 20 µL 10 mM H_2O_2 for UPO activity. For laccase activity, an equal amount of deionized water was added. The assay with shake flask samples was conducted at room temperature for

20 min. For the agar slice assay, 2-mL reaction tubes, containing 500 µL 50 mM sodium citrate buffer (pH 4.5), 250 µL 1.2 mM ABTS, 200 µL ddH_2O, 100 µL 10 mM H_2O_2 (for UPOs) or ddH_2O (for laccases) and one agar slice, were incubated for 1 h at room temperature and 600 rpm. Then, 200 µL reaction mixture were transferred into a 96-well microtiter plate for absorbance measurements using the microplate reader. Product concentration was calculated using the extinction coefficient for oxidized ABTS at 418 nm (ε_{418} = 36 000 $M^{-1}cm^{-1}$) [23]. The respective medium control served as blank (conducted as described in Sect. 2.3.1). For spectrum scans, the buffer was used as blank.

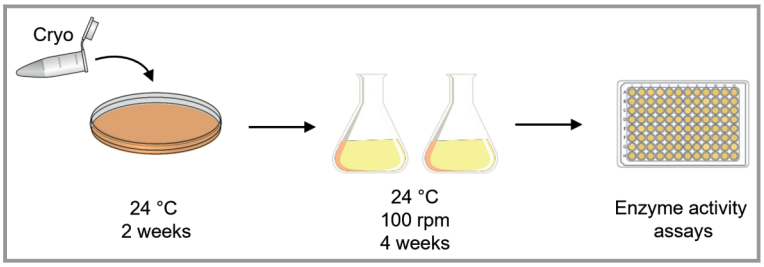

Figure 1. Scheme of the experimental procedure during liquid cultivation. Fungal pre-cultures were inoculated from glycerol stocks and grown on malt extract agar at 24 °C for two weeks. For liquid main cultures, 250-mL shake flasks containing 50 mL carbon- and nitrogen-rich liquid medium were inoculated with a half-overgrown pre-culture plate and cultivated at 24 °C and 100 rpm for four weeks. The fungal mycelium was removed by centrifugation and the crude liquid supernatant was used for the activity assays. NBD was used as an UPO-specific substrate, while ABTS was applied to permit the detection of other oxidoreductases like laccases.

2.4 Validation of 4-NC by HPLC

The analysis of the NBD assay samples was performed with a 1260 Infinity HPLC system (Agilent, USA) using a Nucleoshell RP18 column, 2.0×100 mm, 2.7 µm (Macherey-Nagel, Germany). The flow rate was 0.4 mL min^{-1}. The column temperature was set to 40 °C. The following gradient of 0.1 vol % formic acid (solvent A) and acetonitrile + 0.1 vol % formic acid (solvent B) was used: 0–10 min: 5 to 98 % B; 10–15 min: 98 % B; 15–17 min: 98 to 5 % B. UV detection was performed at a wavelength of 254 nm. For the verification of 4-NC and NBD, external analytical standards (15–500 µM) were measured.

3 Results and Discussion

3.1 Activity Screening during Liquid Cultivation

As well-established UPO workhorses, the two basidiomycetes *A. aegerita* and *C. radians* were chosen for evaluation of the different screening approaches. For a duration of four weeks, fungi were cultured in two carbon- and nitrogen-rich basic liquid media that are known to stimulate the production and secretion of UPOs [24, 25]. For the oxidation assays of enzymes in the culture supernatant, NBD was used as an UPO-specific substrate, while ABTS was applied to permit the detection of other oxidoreductases like laccases. Enzymatic activities were followed by formation of colored 4-NC and ABTS cation radical or dication (Fig. S1, Supporting Information). The experimental procedure is schematically shown in Fig. 1.

Since the background absorbance of the assay mix rose at wavelengths below 430 nm, the absorbance at 514 nm after pH increase was used for product quantification (Fig. S2). However, it should be noted that 4-NC concentrations below approximately 20 µM could not be reliably determined with the UV/Vis spectrometer due to interfering background absorbance. Regarding UPO activity, supernatants of crude culture liquids of *A. aegerita* as well as *C. radians* showed a positive reaction due to formation of 4-NC, indicated by a yellow color at pH 7.0 and red color after alkalization (pH ≥12.0), already after one week of cultivation (Fig. 2). Interestingly, UPO activity of *A. aegerita* was enhanced three-fold while cultivated in GYP medium, in contrast to the widely used soybean meal-containing medium for this strain [4]. Higher product titers were also

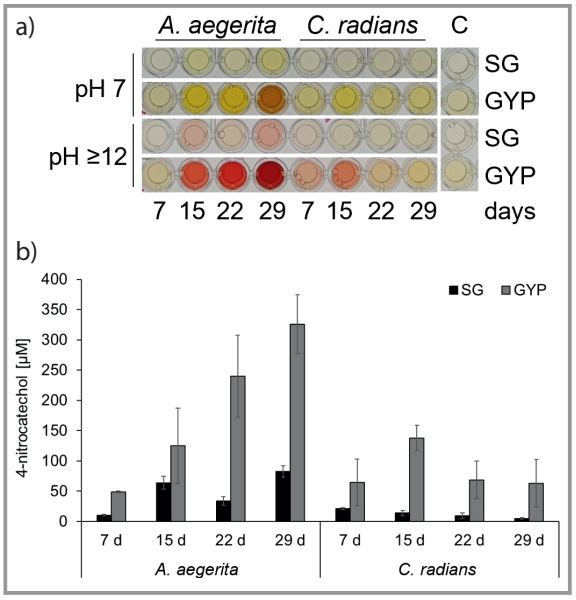

Figure 2. Conversion of NBD by *A. aegerita* and *C. radians*. Parallel measurements of peroxygenase activity during shake flask cultivation of the fungi in a soybean meal (SG) or soy peptone (GYP) medium using the NBD assay [22] (n = 2). The reaction mixture contained 100 µL 100 mM potassium phosphate buffer (pH 7.0), 40 µL 5 mM NBD, 40 µL culture supernatant and 20 µL 10 mM H_2O_2. After 20 min at room temperature, absorbance was measured before and after addition of NaOH using a microplate reader. a) Change of color before (pH 7) and after the addition of sodium lye (pH >12). b) Concentration of 4-NC in the assay mixture followed by absorbance at 514 nm. C: medium control.

observed for *C. radians* in GYP medium, suggesting this medium is more suitable for the expression and secretion of UPOs.

When using ABTS as substrate, visible laccase and UPO activities were found in *A. aegerita* culture supernatant in the absence and presence of H_2O_2 (Fig. 3). Unlike the NBD assay, background absorbance occurred below 390 nm and thus oxidized ABTS could be reliably quantified at 418 nm (Fig. S3). UPO-derived activity was observed throughout the period as the addition of H_2O_2 resulted in increased product formation. The activity of oxidative enzymes in the culture supernatant was significantly higher when *A. aegerita* was cultivated in SG medium. In accordance with the NBD assay, ABTS oxidation with H_2O_2 supply increased over time of cultivation in GYP medium leading to the assumption that the reaction was mainly based on UPO activity. However, a clear distinction between UPOs and other oxidoreductases could not be made in this context.

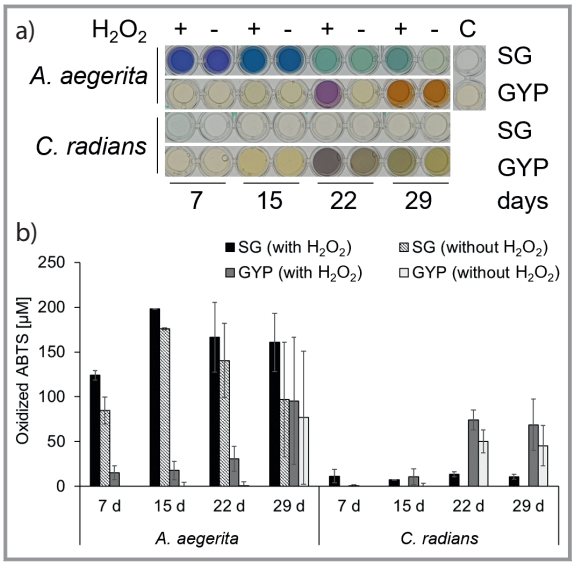

Figure 3. Oxidation of ABTS in culture supernatants of *A. aegerita* and *C. radians* cultivations. Parallel measurements of enzyme activity during shake flask cultivation of the fungi in a soybean meal (SG) or soy peptone (GYP) medium using the ABTS assay with and without addition of H_2O_2 (n = 2). The reaction mixture contained 100 µL 50 mM sodium citrate buffer (pH 4.5), 40 µL 1.2 mM ABTS, 40 µL culture supernatant and 20 µL 10 mM H_2O_2 (for UPOs) or ddH_2O (for laccases). After 20 min at room temperature, absorbance was measured using a microplate reader. a) Change of color according to formation of blue and purple ABTS cations. b) Concentration of oxidized ABTS in the assay mixture followed by absorbance at 418 nm (ε_{418} = 36 000 $M^{-1}cm^{-1}$). C: medium control.

The secretion of oxidative enzymes by *A. aegerita* already started after one week of cultivation in both media. In SG medium, UPOs and other oxidoreductases are suspected, whereas in GYP medium only UPOs could be detected as no oxidative activity was present without the addition of H_2O_2. *C. radians* also started secreting UPOs after one week in SG medium, but only slight product formation was observed. After two weeks, further oxidative reactions were detected when cultured in GYP medium. Thus, enzyme expression appeared to be highly dependent on the medium. Despite several attempts to identify substances and conditions that increase UPO protein expression, it remains unclear which mechanisms actually trigger their production and secretion during cultivation of wild-type fungi [6].

Even though enzyme activity could be monitored, this screening strategy comes with some drawbacks when many strains are screened in parallel under different conditions: Sampling can be difficult due to filamentous growth in mycelia or pellets in shake flasks resulting in a lack of supernatant required for the activity assays. Furthermore, the color of the culture frequently changes during fungal growth in a complex medium, which can impede the distinct spectrophotometric detection of the assay product due to high background absorbance (e.g., Fig. 2a, 3a and S2). Additionally, sufficient cultivation space and equipment are required for long-term cultivation of up to four weeks, especially when different conditions such as media components, temperature, or pH should be tested. Miniaturization of the screening in microtiter plates was not possible due to evaporation of the limited culture volume during the long cultivation period. Therefore, activity screening with fungi grown on agar plates might be a more useful and space-saving approach.

3.2 Activity Screening during Agar Plate Cultivation

In a second step, the screening for enzyme activity was performed by culturing fungi on complex agar media containing soybean meal. Various simple agar plate tests are described in the literature, in which indicator substances such as ABTS are added to the medium and converted by the growing fungus [26]. However, UPO-specific reactions cannot be visualized since these oxidations require the addition of H_2O_2. Therefore, a different strategy was developed. Samples of overgrown agar plates were taken by cutting out agar slices after two and three weeks of incubation, which were then transferred into reaction tubes for the enzyme activity screening (Fig. 4).

After growth on agar plates containing 3 % (w/v) soybean meal, UPO activity was detected for *A. aegerita* as assays showed the formation of 4-NC after 15 and 22 days (Fig. 5 and S4). Furthermore, elevated oxidation of ABTS with H_2O_2 compared to oxidations without H_2O_2 were obtained (Fig. 6 and S5). Surprisingly, no NBD conversion could be observed for *C. radians* cultures. Consequently, the growth on solid medium and the resultant morphological changes probably led to altered enzyme expression profiles. This influence should be considered for future screenings, as false negative strains could be sorted out by this approach.

Nevertheless, in addition to the two model organisms, the applicability of this screening approach was also tested with six other filamentous fungi: *Bipolaris victoriae, Bipolaris*

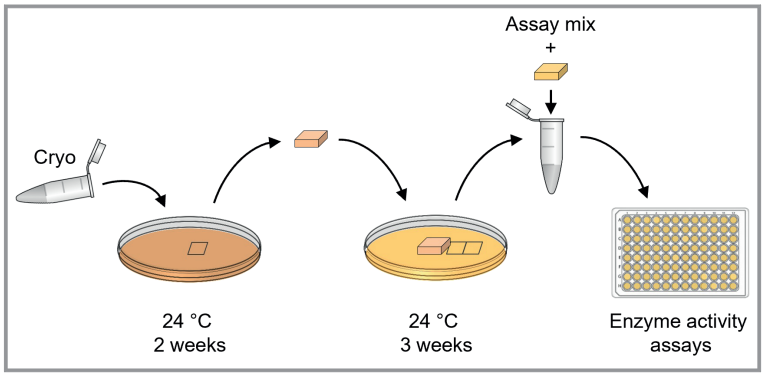

Figure 4. Scheme of the experimental procedure during agar plate cultivation. Fungal pre-cultures were inoculated from glycerol stocks and grown on malt extract agar at 24 °C for two weeks. An overgrown agar slice was used to inoculate main culture plates. Cultures were incubated at 24 °C for three weeks. Overgrown agar slices were used for the activity assays using NBD and ABTS as substrates.

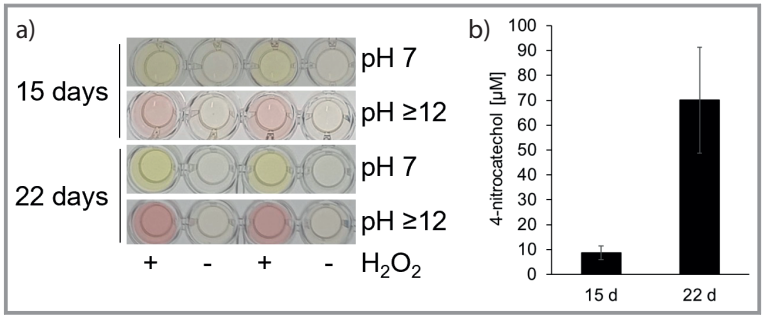

Figure 5. Conversion of NBD by *A. aegerita*. Parallel measurements of enzyme activity using the NBD assay on days 15 and 22 of agar plate cultivation of the fungus growing on 3 % (w/v) soybean meal agar (n = 2). The reaction mixture contained 500 µL 100 mM potassium phosphate buffer (pH 7.0), 200 µL 5 mM NBD, 200 µL ddH_2O, 100 µL 10 mM H_2O_2 and one agar slice in a 2-mL reaction tube. Tubes were incubated for 1 h at room temperature and 600 rpm, 200 µL of reaction mixture were transferred into a 96-well microtiter plate for absorbance measurements without and with NaOH in the microplate reader. a) Change of color before (pH 7) and after the addition of sodium lye (pH >12) with and without addition of H_2O_2. b) Concentration of 4-NC in the assay mixture followed by absorbance at 514 nm.

zeicola, Caldariomyces (Leptoxyphium) fumago, Neofusicoccum parvum, Pyrenophora tritici-repentis, and *Talaromyces stipitatus*. These fungal strains were identified as promising candidates by genome mining and screening for P450s conducted by Schmitz et al. [27]. CPO-expressing fungus *C. fumago* was added as reference strain. Moreover, matches per database entry were counted to determine the number of putative UPO and laccase genes encoded per strain. The conversed heme haloperoxidase family profile ('PS51405') as well as multicopper oxidase signatures ('PS00079'; 'PS00080') that are deposited on PROSITE were matched with protein sequences stored on the protein database UniProtKB. As all five fungi harbored putative UPO sequences (Tab. S1), they were screened for enzyme activity during solid growth. However, none of them converted NBD to 4-NC, but all showed a positive reaction towards oxidation of ABTS without H_2O_2 (Fig. 6b). Due to interfering background absorbance, the insufficient sensitivity of the UV/Vis spectrophotometer measurement for the detection of low product concentrations could be a possible explanation for the absence of formed 4-NC. In contrast, increased oxidation of ABTS with H_2O_2 were obtained for the two reference strains *A. aegerita* and *C. radians* as well as for *P. tritici-repentis* and *T. stipitatus*, compared to oxidations without H_2O_2. To validate this result, the strains were cultivated in liquid culture and subsequently assayed with 4-NC for UPO activity. Interestingly, the crude culture liquid of *T. stipitatus* showed UPO activity after one week of cultivation due to the formation of 4-NC validated by an external 4-NC standard in HPLC analysis (Fig. S6). *P. tritici-repentis* showed no activity, which may be due to either insufficient product titers or inadequate cultivation conditions.

To conclude, it could be shown that the plate-based assay is suitable as a pre-screening tool of fungi to identify suitable candidate strains. In future, this approach can be used for the pre-screening of wild-type fungi from larger strain collections to restrict the library and screen only very suitable candidates for their oxidation potential, with the goal to discover new UPOs of interest for pharmaceutical biotechnology.

4 Conclusion

UPO expression of wild-type fungi is a time-consuming procedure, as secretion often takes several days or even weeks to occur [4, 24, 25]. However, these heme-thiolate enzymes possess a great application potential by transferring an oxygen atom from hydrogen peroxide to numerous substrates. Despite their distribution throughout the whole kingdom of fungi, the availability of UPOs for potential biotechnological applications is restricted due to limitations in the identification of novel UPOs, their production, and the description of their properties [5]. To address this gap, it is of great interest to develop a useful screening strategy of wild-type fungi to identify and characterize putative UPOs. Here, a new approach was presented highlighting the applicability of a screening strategy with solid cultivation and subsequent UPO assay using cell culture samples. The assay was successfully established using reference strains and could be transferred to a small collection of new strains. Thus, a fungus was identified that exhibited UPO-like activity and was not previously known to secrete UPOs. Nevertheless, the presence of an UPO must be verified by heterologous expression of the putative gene or purification of the enzyme. In the future, this may

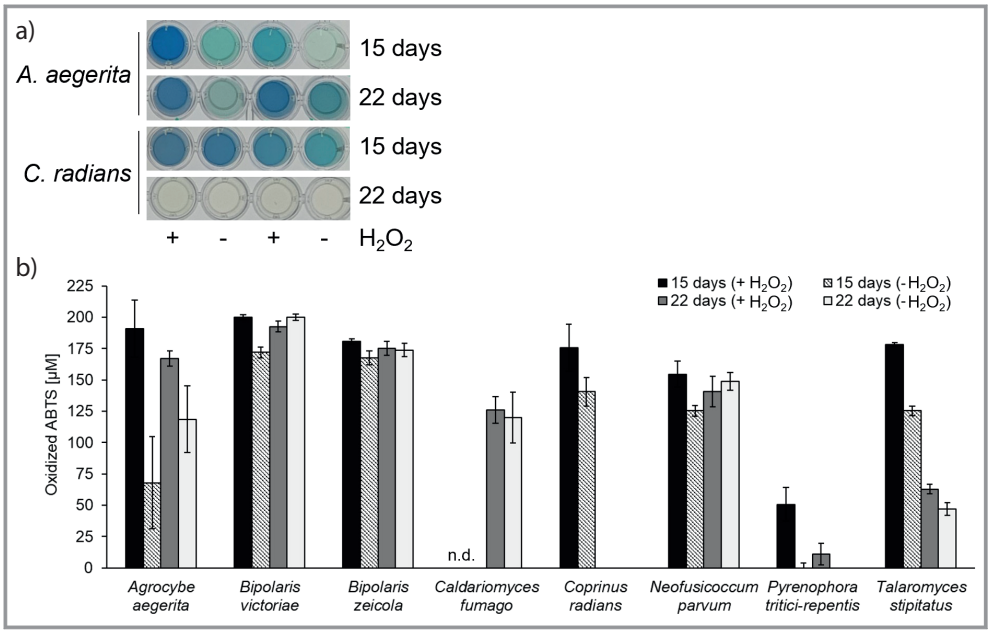

Figure 6. Oxidation of ABTS by *A. aegerita, C. radians* and six test strains. Parallel measurements of enzyme activity using the ABTS assay with and without H_2O_2 on days 15 and 22 of agar plate cultivation of the fungi growing on 3 % (w/v) soybean meal agar (n = 2). 2-mL reaction tubes, containing 500 µL 50 mM sodium citrate buffer (pH 4.5), 250 µL 1.2 mM ABTS, 200 µL ddH_2O, 100 µL 10 mM H_2O_2 (for UPOs) or ddH_2O (for laccases) and one agar slice, were incubated for 1 h at room temperature and 600 rpm. Then, 200 µL of reaction mixture were transferred into a 96-well microtiter plate for absorbance measurements in the microplate reader. a) Change of color according to formation of blue and purple ABTS cations in samples of *A. aegerita* and *C. radians*. b) Concentration of oxidized ABTS in the assay mixture followed by absorbance at 418 nm (ε_{418} = 36 000 $M^{-1}cm^{-1}$). n.d.: not determined.

contribute to the exploitation of novel UPOs as biocatalysts for biotechnological applications.

Supporting Information

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Abbreviations

ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
GYP	Soy peptone medium
HPLC	High performance liquid chromatography
NBD	5-Nitro-1,3-benzodioxol
SG	Soybean meal medium
UPO	Unspecific peroxygenase
4-NC	4-Nitrocatechol

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