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Biocatalytic Screening of the Oxidative Potential of Fungi Cultivated on Plant-Based Resources

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Abstract: The environmental impacts of the postindustrial era, which rely on fossil fuels, have compelled a reconsideration of the future of energy and chemical industries. Fungi are a valuable resource for improving a circular economy through the enhanced valorization of biomass and plant waste. They harbor a great diversity of oxidative enzymes, especially in their secretome. Enzymatic breakdown of the plant cell wall complex and lignocellulosic biomass yields sugars for fermentation and biofuel production, as well as aromatic compounds from lignin that can serve as raw materials for the chemical industry. To harness the biocatalytic potential, it is essential to identify and explore wild-type fungi and their secretomes. This study successfully combined genome mining and activity screening to uncover the oxidative potential of a collection of underexploited ascomycetes and basidiomycetes. The heme peroxidase and laccase activities of four promising candidates, *Bipolaris victoriae*, *Colletotrichum sublineola*, *Neofusicoccum parvum* and *Moesziomyces antarcticus*, were investigated to gain a deeper insight into their enzyme secretion. Furthermore, a plant-based medium screening with the phytopathogen *C. sublineola* revealed that soybean meal is a beneficial component to trigger the production and secretion of enzymes that catalyze H_2O_2-dependent oxidations. These results demonstrate that understanding fungal secretomes and their enzymatic potential opens exciting avenues for sustainable biotechnological applications across various industries.

Keywords: heme peroxidases; laccases; oxidoreductases; wild-type fungi; secretome; genome mining; strain screening; soybean meal; cornmeal; sawdust

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1. Introduction

The industrial production of a wide range of chemicals is dependent upon the availability of fossil fuel resources. The depletion of these finite resources coupled with the alarming environmental consequences of their use has compelled the reconsideration of the future of energy and chemical industries. The development of new technologies is essential to meet human needs for chemical products while utilizing renewable resources in place of persistent and hazardous materials. Plant biomass is considered a carbon-neutral resource. It is, therefore, an attempt to strategically utilize non-edible by-products and waste products derived from forestry and agriculture as renewable feedstocks [1]. In this context, fungi are gaining increasing importance in biotechnology and industry [2]. Hyde et al. described 50 potential uses of fungi, ranging from human medicine and agriculture to the food and waste industry [3]. As fungal survival relies on the uptake of nutrients from the environment, they secrete a series of enzymes to enhance the bioavailability of these compounds [4]. The enzymatic breakdown of lignocellulosic biomass would provide sugars for fermentation and biofuel production, as well as aromatic compounds from lignin, serving as raw material for the chemical industry [5,6]. However, the biodegradation of lignin is challenging because of its water-insoluble, heteropolymeric network formed by phenylpropanoid units. Wood-rotting, saprotrophic fungi, particularly those in the

phyla *Basidiomycota* and *Ascomycota*, secrete significant amounts of lignocellulose-degrading enzymes, making them an important part of the ecosystem [7,8]. It is estimated that wood-decaying basidiomycetes decompose approximately 120 tons of wood per square kilometer per year, releasing the bound carbon and nutrients [9]. Depending on their mode of living, ligninolytic fungi prefer to grow in living plants as pathogens or in animal droppings, the soil, plant litter, and deadwood. White-rot basidiomycetes use a set of oxidative enzymes to attack the lignin moiety of lignocellulose, including laccases (EC 1.10.3.2), lignin peroxidases (LiPs, EC 1.11.1.14), manganese peroxidases (MnPs, EC 1.11.1.13), and versatile peroxidases (VPs, EC 1.11.1.16). Brown-rot basidiomycetes and soft-rot ascomycetes mainly depolymerize cellulose and hemicellulose with hydrolytic enzymes [8,10]. In addition, a certain role in lignin modification is suspected for unspecific peroxygenases (UPOs, EC 1.11.2.1) and dye-decolorizing peroxidases (DyPs, EC 1.11.1.19) [11]. Due to the usage of H_2O_2 as a cosubstrate, fungi also secrete peroxide-supplying enzymes, like aryl alcohol oxidase (EC 1.1.3.7), alcohol oxidase (EC 1.1.3.13), glyoxal oxidase (EC 1.2.3.15), glucose oxidase (EC 1.1.3.4), and pyranose oxidase (EC 1.1.3.10) [12,13]. In case of a phytopathogenic lifestyle, fungi have evolved diverse strategies to break down the plant cell barriers [14]. Secreted cutinases hydrolyze cutin polymers on the plant epidermis, while cellulose, pectin and other compounds in the cell wall are decomposed by several carbohydrate-active enzymes (CAZymes) [15,16]. Secreted peroxidases are thought to play a role in overcoming the initial plant defenses, allowing the fungal hyphae to tolerate environments with high oxidative stress, such as photosynthetically active leaves, or wood decomposition of necrotrophic fungi [15,17].

Due to the diverse biocatalytic capabilities of fungi, it is of great interest to make their catalytic potential available for biotechnological applications. The discovery of new whole-cell biocatalysts or enzymes can be carried out by cultivating naturally occurring microorganisms or by heterologous expression of genes previously identified as promising, with both methods having advantages and disadvantages. Cultivating wild-type fungi under standard lab conditions is a challenge, as the secretome and its mechanisms are not completely understood and enzyme secretion varies greatly depending on the cell type, life cycle phase, as well as environmental conditions, like growth substrate, temperature, and growth phase [15,18]. Nevertheless, the cultivation of wild-type fungi is worth pursuing to fully exploit their potential. For example, Liers et al. used the jelly fungus *Auricularia auricula-judae* to produce and characterize a DyP with an activity up to 8000 U L^{-1} during growth in tomato juice medium [19]. Moreover, fungi are capable of growing on a wide range of organic substrates and thus protein production can be combined with the re-use of agricultural waste, such as brewers' spent grains, wheat straw, tea residues, or sorghum bagasse [20]. For instance, the white-rot fungus *Trametes versicolor* produced laccases with an activity of 2600 U g^{-1} in solid-state fermentation on pretreated corn stalks at 28 °C after 13 days [21].

This study aimed to expand the biocatalytic toolbox of native fungi by activity screening of extracellular heme peroxidases and laccases during growth in different liquid media containing simple or complex plant-based growth substrates. Initial genome mining was used to discover putative heme peroxidase- and laccase-positive fungi in a selected strain collection. A basic medium screening approach demonstrates the importance of growth substrate selection for enzyme production in wild-type fungi. The results of our study indicated that not only saprotrophic fungi, but also phytopathogenic fungi, are worthy of further investigation as potential biocatalysts for oxidative processes, e.g., the degradation of organic materials or detoxification of industrial waste. Thus, the genome mining-assisted screening of wild-type fungi is a promising approach to explore nature's catalytic potential.

2. Materials and Methods

2.1. Genome Mining

Genomes of 64 fungi were mined for secreted oxidoreductases using the protein databases UniProt Knowledgebase (UniProtKB) and PROSITE. PROSITE entries were matched with protein sequences stored on UniProtKB (Table 1).

Table 1. PROSITE signatures and profiles of secreted oxidoreductases for genome mining on UniProtKB.

Putative Enzyme	PROSITE Entry	PROSITE Description
DyP	PS51404	DyP-type peroxidase family
Heme peroxidase	PS00435 PS00436	Peroxidases proximal heme-ligand signature Peroxidases active site signature
Laccase	PS00079 PS00080	Multicopper oxidases signature 1 Multicopper oxidases signature 2
UPO	PS51405	Heme haloperoxidase family profile

2.2. Fungal Strains

Acremonium chrysogenum (DSM 880), *Aureobasidium melanogenum* (CBS 110374), *Aureobasidium pullulans* EXF-150 (DSM 3042), *Aureobasidium subglaciale* EXF-2481 (CBS 123387), *Bipolaris victoriae* FI3 (DSM 62621), *Bipolaris zeicola* 26-R-13 (CBS 237.77), *Colletotrichum fioriniae* PJ7 (CBS 126508), *Colletotrichum gloeosporioides* Nara gc5 (DSM 62136), *Colletotrichum graminicola* M1.001 (CBS 130836), *Colletotrichum higginsianum* (IMI 349063), *Colletotrichum orbiculare* 104-T (CBS 514.97), *Colletotrichum sublineola* (CBS 13130.1), *Cyphellophora europaea* (CBS 101466), *Eutypa lata* UCR-EL1 (CBS 208.87), *Fibulorhizoctonia psychrophila* MB501325 (CBS 109695), *Metarhizium robertsii* ARSEF 23 (MYA-3075), *Mixia osmundae* MB301232 (CBS 9802), *Moesziomyces antarcticus* MB812714 (CBS 516.83), *Moniliophthora roreri* MCA 2997 (CBS 199.77), *Neofusicoccum parvum* UCRNP2 (CBS 133503), *Penicillium oxalicum* 114-2 (DSM 898), *Penicillium roqueforti* FM164 (CBS 221.30), *Podospora anserina* S28 (DSM 980), *Pyrenophora tritici-repentis* Pt-1C-BFP (CBS 265.80), *Sporisorium reilianum* MB323849 (CBS 131459), *Talaromyces stipitatum* ATCC 10500 (CBS 375.48), *Trichoderma virens* Gv29-8 (MYA-4894), *Ustiliago maydis* UM521 (DSM 14603), and *Verticillium alfalfae* VaMs.102 (CBS 127169) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany), and the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands).

2.3. Shake Flask Cultivation of Fungi

Malt extract agar plates (MEA; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were inoculated from glycerol stocks and fungi were grown at 24 °C for two weeks. For liquid main cultures, 50 mL soybean meal- and soy peptone-based liquid medium were inoculated with one half of an overgrown pre-culture plate in 250 mL shake flasks and cultivated at 24 °C and 100 rpm for four weeks in two media. Soybean meal-based medium (SG) consisted of soybean meal (30 g L^{-1}) (Hensel Voll-Soja, Walther Schoenenberger Pflanzensaftwerk GmbH & Co. KG, Magstadt, Germany) and glucose (40 g L^{-1}). Soy peptone-based medium (GYP) contained glucose (42 g L^{-1}), yeast extract (4.5 g L^{-1}), and soy peptone (48 g L^{-1}). Samples were taken weekly, the cells were separated by centrifugation at 13,000× *g* and 4 °C for 20 min, and the supernatant was analyzed via activity assays.

In a second round of cultivation of selected fungi, different media and culture volumes were selected. In total, 100 mL and 200 mL SG medium were inoculated with one half of an overgrown pre-culture plate (MEA) in a 500 mL or 1 L shake flask, respectively, and cultivated at 24 °C and 100 rpm for three to four weeks. For the medium screening, 50 mL medium was inoculated in a 250 mL shake flask as described above. Soybean meal (S) and cornmeal media (C) consisted of 30 g L^{-1} soybean meal and cornmeal (Demeter Bauckhof

Maismehl, Bauck GmbH, Rosche, Germany), respectively. The birch sawdust medium (BS) contained 30 g L^{-1} birch sawdust (Approx. particle size 1 mm × 2 mm; untreated joinery waste, TU Dortmund University, Dortmund, Germany), 5 g L^{-1} soy peptone, 3.2 mM $MgSO_4$, and 0.1% (*v*/*v*) US^{FE} trace elements solution (8.87 g L^{-1} $FeSO_4$ ·7 H_2O, 4.12 g L^{-1} $CaCl_2$ ·2 H_2O, 1.5 g L^{-1} $MnCl_2$ ·4 H_2O, 1.87 g L^{-1} $ZnSO_4$ ·7 H_2O, 0.3 g L^{-1} H_3BO_3, 0.25 g L^{-1} Na_2MoO_4 ·2 H_2O, 0.15 g L^{-1} $CuCl_2$ ·2 H_2O, 0.84 g L^{-1} Na_2EDTA ·2 H_2O, 82.81 mL L^{-1} 37% (*v*/*v*) HCl). Samples were taken as indicated. The glucose content was monitored using glucose test strips (Medi-Test Glukose, Macherey-Nagel GmbH & Co. KG, Düren, Germany).

For the determination of the cell wet weight, cell cultures of *A. pullulans*, *A. subglaciale* and *M. antarcticus* were harvested by centrifugation for 15 min at 18 °C and 3000× *g*. The mycelium of the other fungal strains was harvested by separation through a sieve.

2.4. ABTS Agar Plate Screening

The screening was conducted based on [22]. Fungal pre-cultures were inoculated from glycerol stocks and grown on MEA at 24 °C for two weeks. To screen for oxidoreductases, a 5 mm × 5 mm × 5 mm overgrown agar slice was cut and transferred to a fresh agar plate, containing soybean meal (30 g L^{-1}), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; 250 mg L^{-1} in 50 mM sodium citrate buffer, pH 4.5) and agar-agar (20 g L^{-1}). Agar plates with 1% (*v*/*v*) sodium citrate buffer instead of ABTS served as negative control. Plates were incubated at 24 °C for four weeks.

2.5. Determination of Enzyme Activity in Fungal Cultivation Supernatants

2.5.1. ABTS Assay

The assay was conducted based on [23]. The assay mixture contained 25 mM sodium citrate buffer (pH 4.5), 0.3 mM ABTS and 40 µL culture supernatant in 96-well microtiter plates (No. 655101, Greiner Bio-One™, Kremsmünster, Austria). The reaction was started by adding 0.5 mM H_2O_2 for peroxidase and peroxygenase activity and absorbance at 418 nm of each well was immediately measured every minute or every 30 s for 15 min at 25 °C using the FLUOstar® Omega microplate reader (BMG Labtech, Ortenberg, Germany). For determination of laccase activity without H_2O_2, an equal amount of deionized H_2O was added. Enzyme activity was verified by the characteristic change of color according to formation of blue-green ABTS cations. Product concentration was calculated using the extinction coefficient for oxidized ABTS (ε_{418} = 36,000 M^{-1} cm^{-1}) [23].

2.5.2. 5-Nitro-1,3-benzodioxole (NBD) Assay

The assay and LC-MS quantification were performed based on [24,25]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM NBD (in 100% acetonitrile) and 40 µL culture supernatant in 96-well microtiter plates (No. 655101, Greiner Bio-One™, Kremsmünster, Austria). The reaction was started by adding 1 mM H_2O_2. After incubation for 20 min at room temperature, the reaction was stopped by addition of 15 µL 5 M NaOH. Assay samples were centrifuged at 21,000× *g* and 18 °C for 10 min and the supernatant was analyzed via LC-MS (Figure S1).

NBD and 4-nitrocatechol were quantified with a 1260 Infinity II LC System coupled to a 6120 quadrupole (Agilent Technologies, Inc., Santa Clara, CA, USA) using a Nucleoshell RP18 column, 2.0 mm × 100 mm, 2.7 µm (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The flow rate was 0.4 mL min^{-1}. The column temperature was set to 40 °C. The following gradient of 0.1% (*v*/*v*) formic acid (solvent A) and acetonitrile + 0.1% (*v*/*v*) formic acid (solvent B) was used: 0–10 min: 5 to 98% B; 10–15 min: 98% B; and 15–17 min: 98 to 5% B. UV detection was performed at a wavelength of 254 nm. The following ESI parameters were set: drying gas temperature: 350 °C, nebulizer pressure: 2.41 bar, drying gas flow: 12 L min^{-1}, capillary voltage: 3000 V. 4-Nitrocatechol was detected in a range of m/z 100 to 1000. For the verification of 4-nitrocatechol (m/z = 156.1, 178.1) and NBD (m/z = 168.1, 190.1), external analytical standards (15–500 mM) were measured.

2.5.3. Veratryl Alcohol Assay

The assay and LC-MS quantification were conducted based on [26,27]. The assay mixture contained 50 mM McIlvaine buffer (pH 7), 1 or 0.5 mM veratryl alcohol (in 100% acetonitrile), and 50 µL supernatant. The reaction was started by adding 1 or 0.5 mM H_2O_2 and microtubes were incubated in an Eppendorf ThermoMixer® C (Eppendorf SE, Hamburg, Germany) at 500 rpm and 21 °C for 30 min. For determination of substrate conversion without H_2O_2, an equal amount of deionized H_2O was added. Then, the assay samples were centrifuged at 20,000× *g* and 18 °C for 10 min and the supernatant was analyzed via LC-MS (Figure S2).

Veratryl alcohol and veratraldehyde were quantified with a 1260 Infinity II LC System coupled to a 6120 quadrupole (Agilent Technologies, Inc., Santa Clara, CA, USA) under the same condition as described above. The following gradient of 0.1% (*v*/*v*) formic acid (solvent A) and methanol + 0.1% (*v*/*v*) formic acid (solvent B) was used: 0–0.5 min: 5% B; 0.5–1 min: 5 to 20% B; 1–10 min: 20 to 50% B; 10–15 min: 50 to 95% B; 15–17 min: 95% B; and 17–20 min: 95 to 5% B. UV detection was performed at a wavelength of 280 nm. The following ESI parameters were set: drying gas temperature: 350 °C, nebulizer pressure: 2.41 bar, drying gas flow: 12 L min^{-1}, and capillary voltage: 3500 V. Veratraldehyde was detected in a range of m/z 100 to 300. For the verification of veratryl alcohol (m/z = 151.1, 152.1, 191.1) and veratraldehyde (m/z = 167.1, 168.1, 189.1), external analytical standards (15–1000 mM) were measured.

2.6. SDS-PAGE Analysis of Culture Supernatants

The supernatant samples of the fungal cultures were concentrated by methanol-chloroform precipitation. In total, 200 µL of culture supernatant, 115 µL methanol and 40 µL chloroform were mixed, vortexed and centrifuged at 16,000× *g* and 18 °C for 5 min. The upper, aqueous phase was discarded, and 72 µL methanol was added, vortexed and centrifuged again under the same conditions. Then, the supernatant was discarded, the pellet was dried for 45 min at room temperature and finally resuspended in 10 µL deionized H_2O resulting in a 20-fold concentration. Concurrently, the supernatant of *M. antarcticus* cultures was filtrated and concentrated using an Amicon® Ultra-15 mL centrifugal filter (50 kDa; Merck KGaA, Darmstadt, Germany). Filtrate as well as retentate were also analyzed for conversion of veratryl alcohol.

Concentrated samples were analyzed via SDS-PAGE (12% acrylamide) utilizing a Bio-Rad Mini-Protean Tetra cell electrophoresis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PageRuler™ Plus Prestained Protein Ladder and PageRuler™ Unstained Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA) were included, covering a molecular weight range between 10 and 250 kDa. Proteins were visualized using a Coomassie brilliant blue R-250 staining solution.

3. Results and Discussion

3.1. Genome Mining as a Tool for Pre-Selection of Promising Fungal Strains

Fungi secrete laccases as well as heme peroxidases, like MnPs, LiPs, VPs, UPOs, and DyPs, which are known or assumed to be involved in the oxidative degradation of lignocellulosic material [11,28]. All enzymes show characteristic amino acid sequence signatures, making genome analysis for the presence of conserved patterns a promising approach to identify strains with high oxidative potential. Thus, fungal genomes were mined by scanning UniProtKB entries against the respective PROSITE entries. In particular, promising fungal strains, selected by Schmitz et al. (33 strains) and Faiza et al. (31 strains), were focused on in this project [29,30]. To determine the number of putative proteins per strain, matches per fungal strain were counted while only hits with a signal peptide were included in the analysis (Figure 1). Nevertheless, it should be noted that this in silico method may not reflect the full potential of the fungi, as secretion of fungal proteins is also possible without a signal peptide [18].

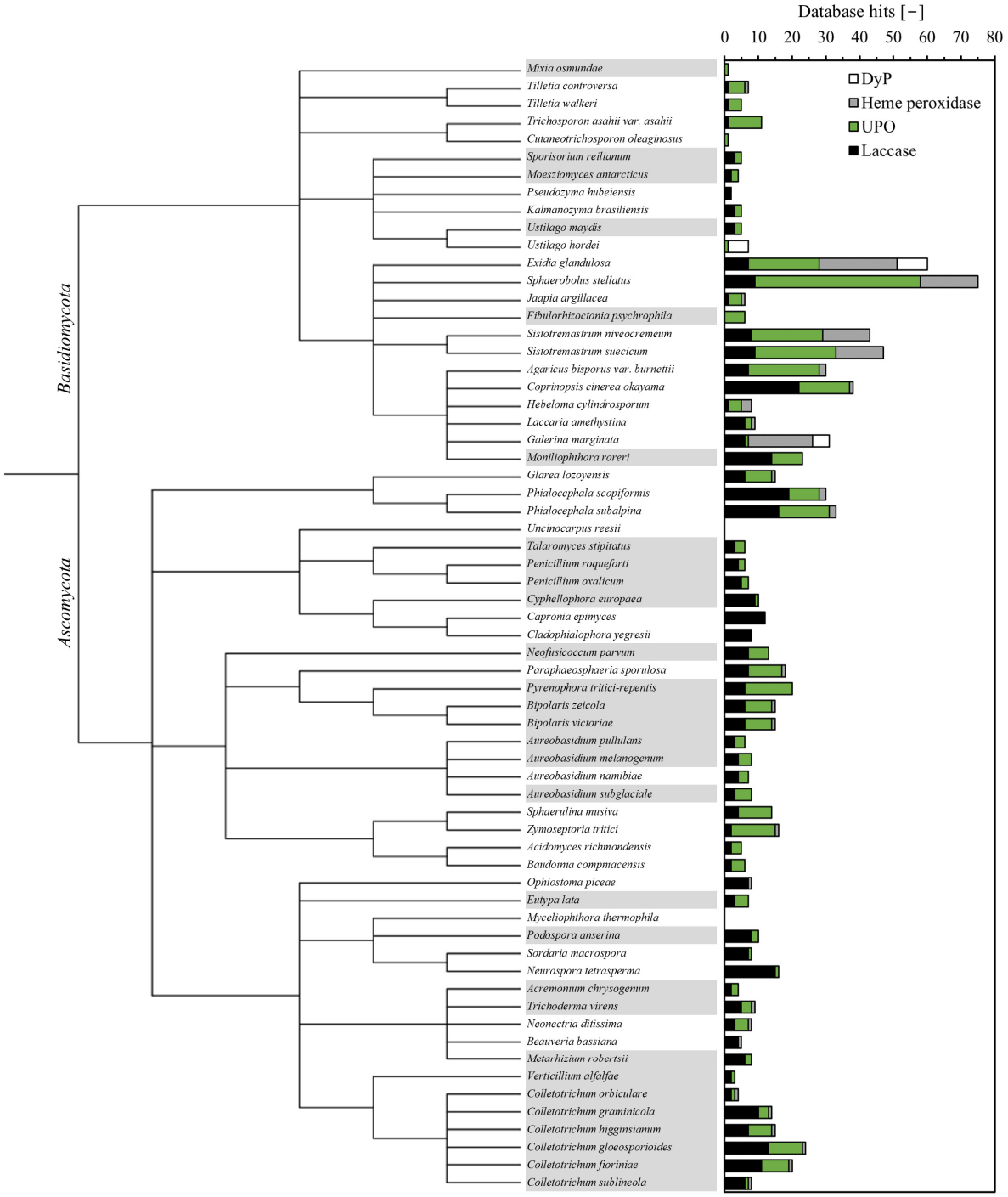

Figure 1. Taxonomic classification of the mined fungi, belonging to the phyla *Ascomycota* and *Basidiomycota*, based on NCBI Common Tree and visualized using the online tool 'iTOL: Interactive Tree Of Life'. PROSITE entries (DyP: PS51404, heme peroxidase: PS00435 + PS00436, Laccase: PS00079 + PS00080, UPO: PS51405) were matched with protein sequences stored on UniProtKB and only sequences containing a signal peptide were considered. Strains highlighted in gray were selected for activity screening during shake flask cultivation. DyP, dye de-colorizing peroxidase; UPO, unspecific peroxygenase.

As white-rot fungi can be found in the class of *Agaricomycetes* in the phylum *Basidiomycota*, it is not surprising that fungi of related orders exhibited numerous putative heme peroxidases and laccases. For instance, *S. stellatus* [31], *F. psychrophila* [32], *A. bisporus* [8], *C. cinerea* [8], and *G. marginata* [32] are referred to as white-rot fungi in the literature with a

mostly high number of database hits in this analysis. *F. psychrophila* is also described as phytopathogenic fungus causing post-harvest diseases of apples and pears [33]. Its pathogenic lifestyle could be the reason for missing classical ligninolytic oxidoreductases discovered in this genome mining since it does not infect woody tissue. In general, false-positive or false-negative hits cannot be avoided in this rather rough database search. For example, Riley et al. showed that the saprophyte *Jaapia argillacea* had wood decay properties similar to certain white-rot fungi, but its genome did not encode the characteristic ligninolytic LiPs, MnPs and VPs. The one class II peroxidase found lacked the catalytic tryptophan and Mn-binding sites and was therefore unlikely to directly modify lignin. Consequently, the authors proposed a reconsideration of the classification of fungal decay modes beyond lignin-degrading heme peroxidases and CAZymes [34].

Most of the ascomycetes listed here have a phytopathogenic lifestyle (Figure 1). As expected, the number of putative heme peroxidases seemed to be lower in plant pathogenic fungi compared to saprotrophic, wood-rotting fungi. The secretion of laccases, MnPs, LiPs, or other cell-wall-degrading enzymes can have two purposes: firstly, they can enable the degradation of lignocellulose to supply carbohydrates, and secondly, they support the penetration and lesion formation [35,36]. In the plant, they are suggested to trigger a number of immune responses as virulence factors, including reactive oxygen species (ROS) burst or programmed cell death [37]. Necrotrophic fungi thrive on dead host tissue, that has been necrotized by the action of secreted cell-wall-degrading enzymes, phytotoxic secondary metabolites, or ROS [38]. The genus *Colletotrichum* and the class *Dothideomycetes*, amongst others, *N. parvum*, *Aureobasidium* spp., *P. tritici-repentis*, and *Bipolaris* spp., possessed mostly UPO- and laccase-like proteins. Numerous necrotrophic *Dothideomycetes* produce phytotoxic metabolites and peptides, that are essential for their pathogenicity [38]. Widely distributed *Colletotrichum* species are endophytes or plant pathogens, which are also known to produce a variety of secondary metabolites with diverse bioactivities [39]. As fungal secretion of detoxifying enzymes, such as catalase and peroxidase, is a critical mechanism to counteract the harmful effects of ROS [40], extracellular UPOs could also play a role in this context.

The genome mining approach provided valuable insights into the secretomes of mostly unexplored fungi, that can be used to categorize and prioritize the given and future strain collections. Finally, 29 fungal strains were selected for a subsequent activity screening with the model substrates ABTS, NBD and veratryl alcohol during shake flask cultivation (Figure 2). The number of candidates was reduced based on different criteria, namely strain availability, cultivability, novelty in this context, and number of databases hits. Schmitz et al. revealed a fungal strain collection as a promising source for new cytochrome P450 monooxygenases [30], of which 23 ascomycetous species and one basidiomycetous species were also likely to harbor oxidative enzymes in their secretome. These fungi were all selected for the strain screening. Additionally, five promising *Basidiomycota* from Faiza et al. were chosen for the screening to have a more diverse collection of fungi [29].

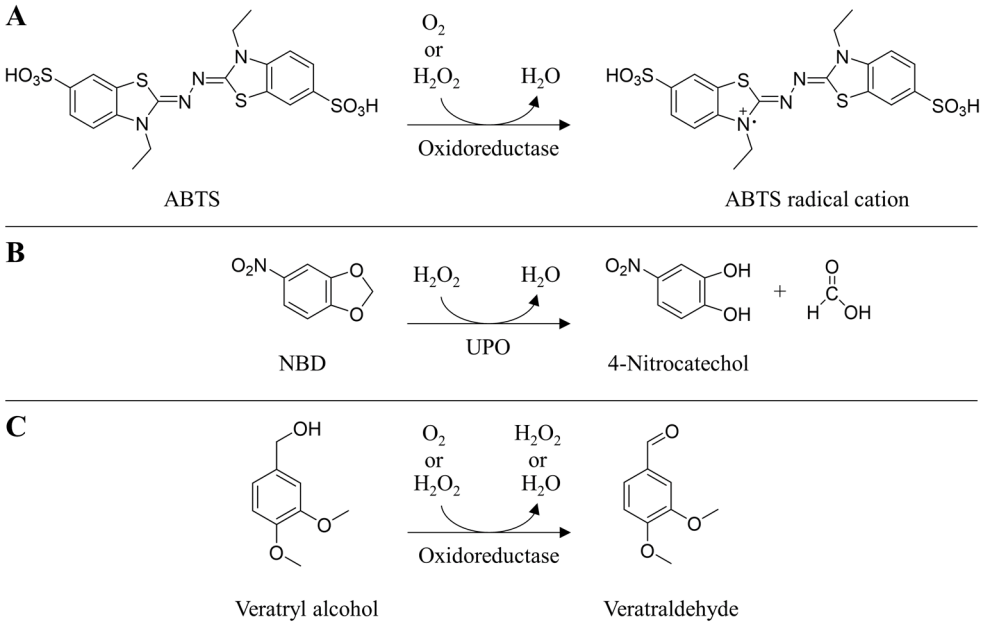

Figure 2. Enzyme assays for the detection and quantification of different oxidoreductase activities by oxidation of ABTS (**A**), NBD (**B**), and veratryl alcohol (**C**). ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); NBD, 5-nitro-1,3-benzodioxole; and UPO, unspecific peroxygenase.

3.2. Enzyme Activity Screening of Fungal Secretomes

To determine the oxidative potential of secreted enzymes, selected fungi from the genome mining approach were cultivated and tested for conversion of the two model substrates ABTS and NBD (Figure 2A,B). Solid growth on nitrogen-rich soybean-ABTS agar plates and liquid cultivation in two different media were chosen to reveal possible differences in the enzyme activity due to morphology- and medium-dependent expression or secretion. Soybean meal-based as well as soy peptone-based media are known to stimulate the secretion of fungal enzymes [41,42]. A constant amount of soybean meal with increasing glucose concentration led to an almost linear increase in *Cra*UPO activity secreted by the coprophilous and litter-decomposing fungus *Coprinus radians* [42]. The most favorable composition with 3% (*w*/*v*) soybean meal and 4% (*w*/*v*) glucose was therefore also chosen in this project. In addition, a carbon- and nitrogen-rich medium was selected, containing 4.2% (*w*/*v*) glucose, 4.8% (*w*/*v*) soy peptone and 0.45% (*w*/*v*) yeast extract, which Gröbe et al. used to successfully produce the *Mro*UPO with the basidiomycete *Marasmius rotula* [41].

Determination of UPO activity was carried out with NBD according to Poraj-Kobielska et al. [24]. Laccase and peroxidase activity was determined with ABTS. Samples of the culture supernatants were taken weekly and analyzed via in vitro assays. ABTS oxidation in agar plates was confirmed by strong coloring from light yellow to dark purple around the fungal mycelium [22].

Seven tested fungi oxidized ABTS to dark purple ABTS cation radicals ($ABTS^{\bullet+}$) during solid growth, indicating the production of oxidoreductases after 7 and 14 days of incubation (Table 2). Except *C. fioriniae* and *P. tritici-repentis*, all these fungi also oxidized ABTS during growth in liquid SG medium. Interestingly, SG medium appeared to promote oxidoreductase secretion better than soy peptone-based GYP medium, in which ABTS oxidation was rarely observed. As the complexity of the carbon source influences the secretome composition, the more complex SG medium might be more beneficial compared to GYP medium [18]. *M. antarcticus*, *C. sublineola* and *F. psychrophila* converted ABTS only in combination with H_2O_2, suggesting the presence of peroxidases or UPOs. The basidiomycete *M. antarcticus* as well as *C. sublineola* and 14 other ascomycetes also oxidized NBD with H_2O_2 to 4-nitrocatechol, which indicated UPO-like activity. A total of 21 out of 29 fungal species showed distinct enzyme activity toward ABTS, NBD, or both substrates resulting in a hit

rate of 72% for the genome mining-based screening approach for wild-type fungal activity. However, the number of presumed protein sequences alone is not a sufficient indicator of the level of enzyme activity (Table 2). Eight strains did not show activity in any assay. Since enzyme expression and secretion of enzymes is highly regulated by environmental factors, such as growth substrate, temperature, and pH, it is possible that unfavorable cultivation conditions were chosen for these pathogenic or coprophilic fungi [15,18].

Table 2. Peroxygenase, peroxidase, and laccase activity screening of fungi during four weeks of shake flask or agar plate cultivation at 24 °C. Oxidation of NBD (marked red if product was found via LC-MS; conducted with H_2O_2) and ABTS (marked green if color change was visible; conducted with and without H_2O_2) by the fungal supernatants were determined weekly. The pH of the shake flask cultures was measured after four weeks. All UniProtKB hits for the PROSITE entries included in this genome mining are shown.

Fungal Strain	Strain Number	Family	Characteristics	Database Hits	Medium	NBD	ABTS			pH
							+ H_2O_2 [a]	− H_2O_2 [a]	Plate [b]	
P. oxalicum	DSM 898	*Aspergillaceae*	Saprotrophic mold	7	SG					5.7
					GYP				-	7.8
P. roqueforti	CBS 221.30		Saprotrophic mold	6	SG					8.6
					GYP				-	8.8
F. psychrophila	CBS 109695	*Atheliaceae*	White-rot fungi/ phytopathogenic	6	SG					8.3
					GYP				-	9.3
N. parvum	CBS 133503	*Botryosphaeriaceae*	Phytopathogenic	13	SG				7	8.7
					GYP				-	8.4
M. robertsii	MYA-3075	*Clavicipitaceae*	Entomopathogenic	8	SG					8.2
					GYP				-	8.2
C. europaea	CBS 101466	*Cyphellophoraceae*	Opportunistic pathogen	10	SG					7.1
					GYP				-	9.1
E. lata	CBS 208.87	*Diatrypaceae*	Phytopathogenic	7	SG					8.3
					GYP				-	8.8
C. fioriniae	CBS 126508	*Glomerellaceae*	Phytopathogenic	20	SG				14	9.1
					GYP				-	9.6
C. gloeosporioides	DSM 62136		Phytopathogenic	24	SG					9.3
					GYP				-	9.4
C. graminicola	CBS 130836		Phytopathogenic	14	SG					8.8
					GYP				-	8.9
C. higginsianum	IMI 349063		Phytopathogenic	15	SG					8.6
					GYP				-	9.1
C. orbiculare	CBS 514.97		Phytopathogenic	4	SG					8.8
					GYP				-	8.8
C. sublineola	CBS 13130.1		Phytopathogenic	8	SG					8.2
					GYP				-	8.7
A. chrysogenum	DSM 880	*Hypocreaceae*	Mold	4	SG					9.2
					GYP				-	9.6
T. virens	MYA-4894	*Hypocreaceae*	Saprotrophic	9	SG					8.5
					GYP				-	8.8
M. roreri	CBS 199.77	*Marasmiaceae*	Phytopathogenic	28	SG				7	6.1
					GYP				-	6.7
M. osmundae	CBS 9802	*Mixiaceae*	Phytopathogenic	1	SG					8.9
					GYP				-	9.4
B. victoriae	DSM 62621	*Pleosporaceae*	Phytopathogenic	15	SG				7	8.8
					GYP				-	9.5
B. zeicola	CBS 237.77		Phytopathogenic	15	SG				7	9.3
					GYP				-	9.3
P. tritici-repentis	CBS 265.80		Phytopathogenic	20	SG				7	8.7
					GYP				-	8.6

Table 2. *Cont.*

Fungal Strain	Strain Number	Family	Characteristics	Database Hits	Medium	NBD	ABTS			pH
							+ H_2O_2 [a]	− H_2O_2 [a]	Plate [b]	
V. alfalfae	CBS 127169	*Plectosphaerellaceae*	Phytopathogenic	3	SG					9.3
					GYP				-	5.5
P. anserina	DSM 980	*Podosporaceae*	Coprophilic	10	SG					9.2
					GYP				-	9.4
A. melanogenum	CBS 110374	*Saccotheciaceae*	Saprotrophic mold	8	SG					8.3
					GYP				-	9.3
A. pullulans	DSM 3042		Saprotrophic mold	6	SG					9.0
					GYP				-	9.5
A. subglaciale	CBS 123387		Polyextremotolerant mold	8	SG					8.2
					GYP				-	9.3
T. stipitatus	CBS 375.48	*Trichocomaceae*	Saprotrophic	6	SG				7	8.6
					GYP				-	8.7
M. antarcticus	CBS 516.83	*Ustilaginaceae*	Phylloplane yeast	4	SG					9.1
					GYP				-	9.2
S. reilianum	CBS 131459		Phytopathogenic	5	SG					8.7
					GYP				-	9.4
U. maydis	DSM 14603		Phytopathogenic	5	SG					8.3
					GYP				-	9.2

-, not determined; [a], ABTS oxidation was evaluated by visible color change from colorless to green after 20 min; [b], dark purple coloring around and below the fungal mycelium after 7 or 14 days growing on soybean meal-agar supplemented with 250 mg L^{-1} ABTS; GYP, glucose (42 g L^{-1}), yeast extract (4.5 g L^{-1}), and soy peptone (48 g L^{-1}) medium; SG, soybean meal (30 g L^{-1}) and glucose (40 g L^{-1}) medium; green, oxidation of ABTS; red, oxidation of NBD to 4-nitrocatechol.

NBD-positive strains were also tested for peroxidase activity using the lignin model compound veratryl alcohol (Figures 2C and 3, Table S1). SG medium was chosen for the second cultivation as soybean meal appeared to trigger enzyme secretion more efficient than the GYP medium. Despite similar inoculation of fungal main cultures, replicates occasionally exhibited enzyme activities and culture states with a slight time shift, resulting in elevated average deviations during activity measurements (Figure 3, Table S1). Mycelial-growing strains achieved a cell wet weight of up to 700 g L^{-1}, while the yeast *M. antarcticus* and the two *Aureobasidium* species grew to a cell wet weight of 100 g L^{-1} (Figure A1A). Five fungi converted veratryl alcohol to veratraldehyde during the entire cultivation period of four weeks, whereby the secretome of *M. antarcticus* exhibited the highest product yield with almost 50% in the first cultivation week. High product concentrations might result from high enzyme activity, a high amount of secreted enzyme, or a combination of several enzymes. The genome mining revealed only four putative protein sequences in the basidiomycetous yeast. Thus, a high number of putative sequences is not indicative of the level of oxidative activity, as other fungi with more sequences showed no activity. Veratryl alcohol conversion of *M. antarcticus* culture supernatant might also be attributed to an aryl-alcohol oxidase (*Ma*AAO), which was identified and expressed in *Pichia pastoris* showing a specific activity toward veratryl alcohol of 25.7 U mg^{-1} [43]. Presumably, *Ma*AAO was secreted by its native producer in this cultivation approach.

In general, the lignin model substrate veratryl alcohol is accepted by LiPs, VPs, DyPs, and UPOs [42,44,45]. To our knowledge, it is not described for MnPs to convert the alcohol. Characterized UPO secretion of wild-type fungi *Cyclocybe aegerita* (formerly *Agrocybe aegerita*), *Coprinellus radians*, *Marasmius rotula*, *Marasmius wettsteinii*, *Chaetomium globosum*, and *Truncatella angustata* begins a few days after inoculation and peaks between two and three weeks of cultivation depending on the fungus [26,41,42,46–48]. As UPO activity in the secretomes often significantly declines after three weeks, it is not unexpected that the NBD-oxidizing activity of the strains described here did not remain constant throughout

the entire cultivation period. Various wood-rotting fungi showed a similar course of activity for laccases, MnPs, and LiPs with maxima around the 10th day [49,50]. In contrast, the secretion of the DyP produced by *A. auricula-judae* reached its maximum level approximately four weeks after inoculation [19]. Consequently, the constant conversion of veratryl alcohol observed over the four-week period could be the result of the combined activity of different oxidizing enzymes secreted at different times during the cultivation process.

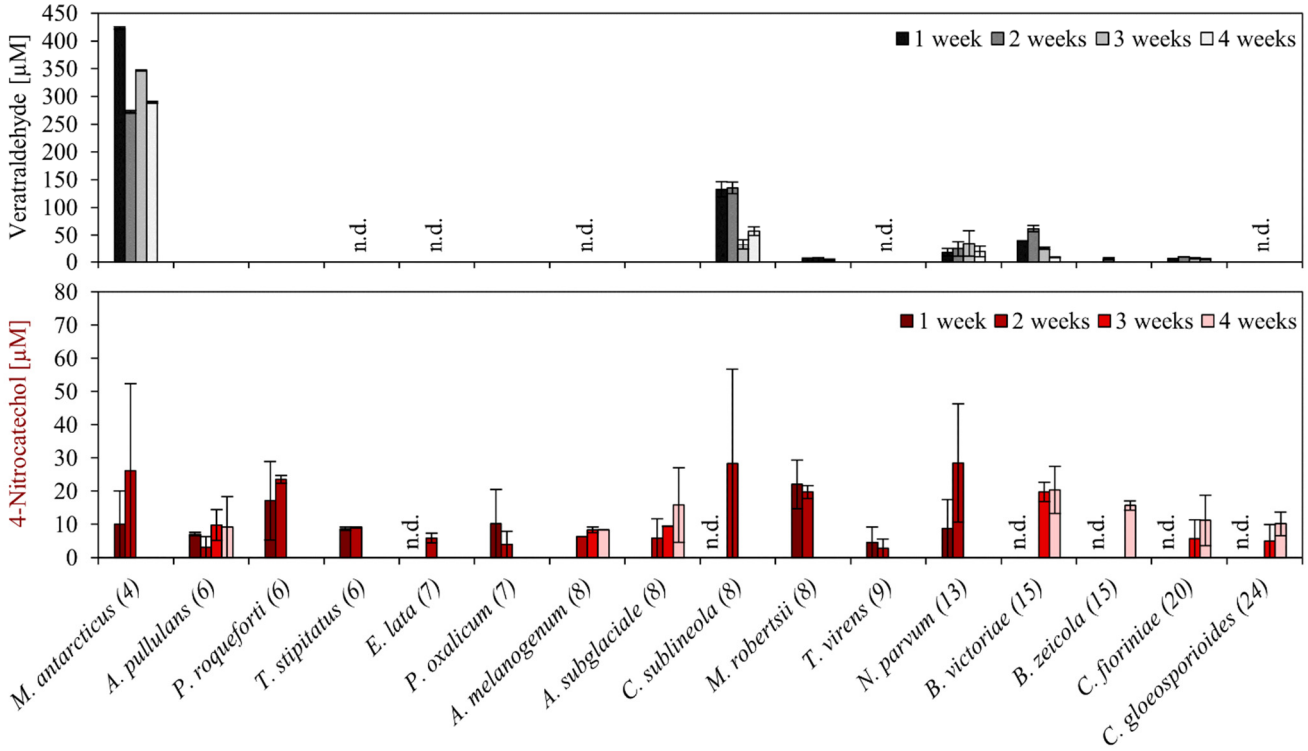

Figure 3. Product concentration of veratraldehyde and 4-nitrocatechol. Values given are means and average deviations for duplicates. Peroxidase and peroxygenase activity were determined by the oxidation of 1 mM veratryl alcohol to veratraldehyde (black gradation) for 30 min and 1 mM NBD to 4-nitrocatechol (red gradation) for 20 min with 1 mM H_2O_2 using culture supernatant during 250 mL shake flask cultivation of the fungi in SG medium at 24 °C for four weeks. The assays were each performed during two different cultivations, in which the fungi were cultivated in duplicates. Due to solid growth of the fungi, some samples could not be taken during the cultivation (marked with n.d.). Product concentration was quantified by LC-MS using an external standard (15–500 µM). All UniProtKB hits for the selected PROSITE entries of this genome mining are shown in brackets. n.d., not determined.

In conclusion, this activity screening revealed some underexploited wild-type fungi as interesting enzyme producers, which probably secrete ligninolytic enzymes and should therefore be described in more detail in further cultivation approaches. Contrary to expectations, the use of GYP medium did not have a beneficial effect on enzyme secretion.

3.3. Detailed Activity Profile of Four Veratryl Alcohol-Positive Strains

Due to their ability to oxidize veratryl alcohol and NBD, *B. victoriae*, *C. sublineola*, *M. antarcticus*, and *N. parvum* proved to be promising candidates for the secretion of oxidative enzymes. To gain a deeper insight into the course of enzyme secretion, these four fungi were cultivated again in 500 mL shake flasks for 22 days under the same conditions as before. Enzyme activity was monitored by conversion of veratryl alcohol and ABTS with and without the addition of H_2O_2 (Figure 4A,B). Secreted enzymes of the endophyte *N. parvum* oxidized ABTS with a volumetric activity of about 30 U L^{-1} and a constant

product yield of up to 89% after day 12 of cultivation. Recently, a secretome analysis of *N. parvum* identified a putative extracellular laccase, which could be responsible for the ABTS-oxidizing activity observed here [51]. In contrast, the basidiomycetous *M. antarcticus* showed almost no conversion of ABTS. However, *M. antarcticus* converted veratryl alcohol with a constant product yield of 25%, while *N. parvum* did not exhibit high yields for veratraldehyde. Interestingly, almost all strains reached comparable product yields with and without H_2O_2 for both assays. Only *C. sublineola* appeared to secrete H_2O_2-dependent enzymes, which oxidized ABTS with a volumetric activity of 20 U L^{-1} and 61% product yield. Consequently, peroxidases or peroxygenases are more likely to be responsible for ABTS oxidation rather than laccases using O_2 as a cosubstrate.

In both assays, secreted enzymes of ascomycetous *B. victoriae*, which causes Victoria blight of oats and related grains, achieved a moderate product yield of around 10–30%. As the activity of enzymes toward veratryl alcohol and ABTS followed a similar pattern throughout the entire cultivation period, it can be inferred that the expression and secretion of the responsible enzymes were induced simultaneously (Figure 4A). Regarding UPOs, their production often coincides with laccase secretion [26,41]. Comparable activities were observed for *B. victoriae*, *C. sublineola*, and *N. parvum* in this cultivation. The pH increased considerably in all cultures during the cultivation period of three weeks, a tendency also observed for *Coprinus* species cultivated in a similar medium [42]. An increase in protein or peptide degradation leads to the release of ammonia and an elevation of the pH [52]. Soybeans have a protein content ranging from 39% to 44% [53]. Therefore, an alkalization of the medium due to fungal degradation can be expected. Cultures of *N. parvum* also turned black after one week of cultivation, which is consistent with descriptions previously published for this fungus (Figure 4D) [54]. The observed pigmentation of the culture might have interfered with the absorbance measurements during the ABTS assay, resulting in occasional high average deviations.

To obtain an overview of the range of secreted proteins, SDS-PAGE analysis of the culture supernatants was performed (Figure 4C). Samples from earlier time points in the cultivation period resulted in a smear in the SDS gel due to the high protein content of soybeans. The SDS gel of *M. antarcticus* culture supernatant revealed two distinct bands with a molecular mass of around 40 and 70 kDa. Previously described *Ma*AAO from *M. antarcticus* has a theoretical mass of 67 kDa and the glycosylated enzyme exhibited a mass of 75 kDa [43]. Hence, these two protein fractions were separated by ultrafiltration with a cut-off membrane of 50 kDa. Conversion of veratryl alcohol was significantly decreased in the filtrate compared to the retentate. Thus, it is very likely that *Ma*AAO was produced by *M. antarcticus* under the cultivation conditions chosen here. It is also possible that the distinct band around 40 kDa belonged to a heme peroxidase or an UPO with a predicted mass of 42 and 43 kDa, respectively (Table S2). Genome mining identified 15 putative protein sequences in *B. victoriae*, of which eight heme peroxidase-like proteins have a predicted molecular mass ranging from 31 to 48 kDa. Due to post-translational modifications, such as glycosylation, the protein mass is most likely larger than the predicted mass. Proteins in the range of 30 to 55 kDa could therefore possibly correspond to one or more genome-mined proteins. Considering the potential loss of protein during the concentration procedure, all strains appeared to secrete a consistent protein profile throughout the cultivation process.

In general, the fungi reached a similar cell wet weight as before (Figure A1B). Hence, the cultivation of these four wild-type fungi led to reproducible results as well as interesting novel insights in the oxidative activity of their secretomes. As the phytopathogen *C. sublineola* exhibited clear H_2O_2-dependent enzyme activity toward ABTS, that had not been previously described, this strain was selected for activity and growth screening on different liquid media containing complex plant-based carbon and nitrogen sources.

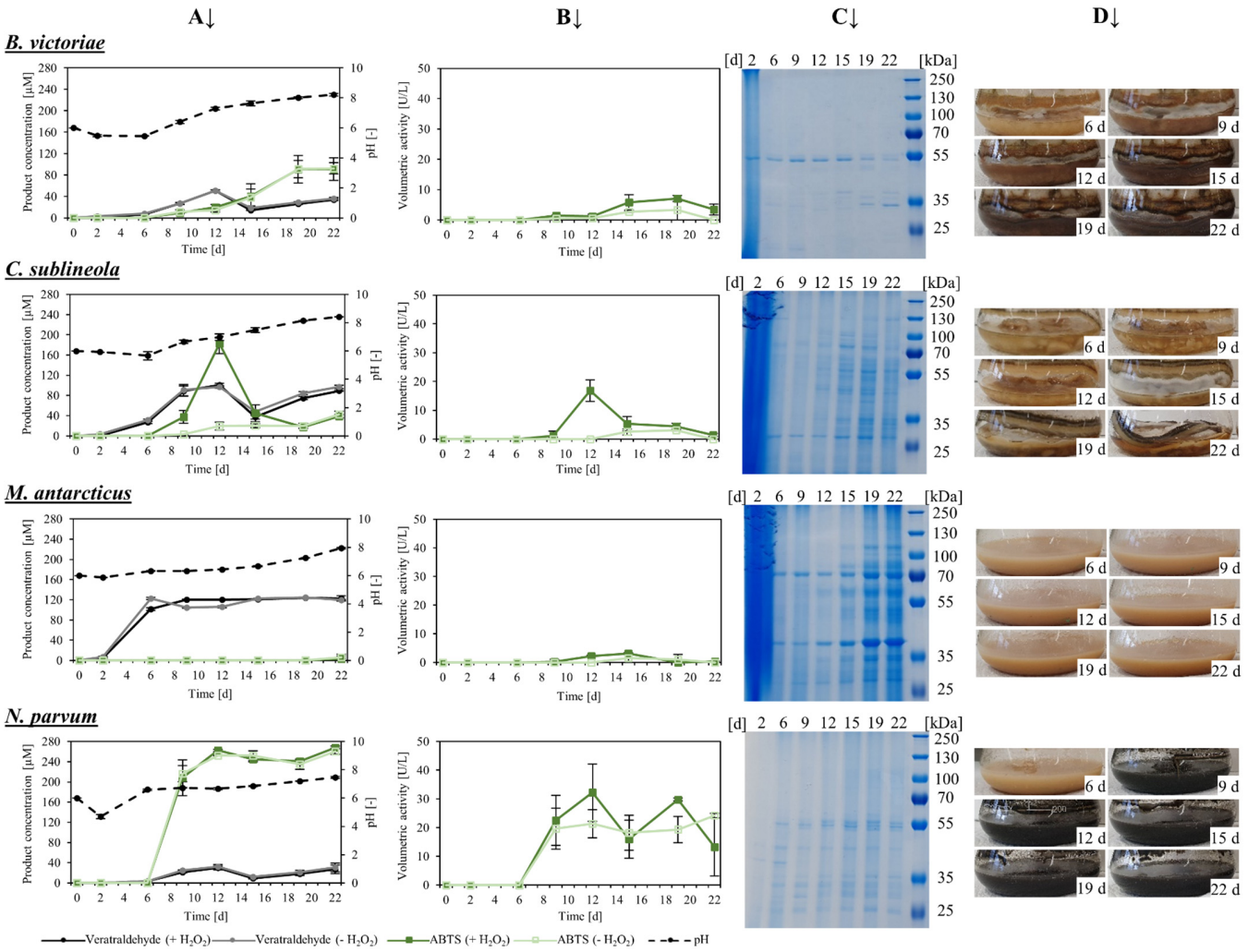

Figure 4. Shake flask cultivation of *B. victoriae*, *C. sublineola*, *M. antarcticus*, and *N. parvum* in SG medium. Values given are means and average deviations for duplicates. (**A**) pH of the culture supernatant (dashed line) and conversion of 0.5 mM veratryl alcohol to veratraldehyde (circles; with H_2O_2 in black, without H_2O_2 in grey) for 30 min at 21 °C and oxidation of 0.3 mM ABTS (rectangles; with H_2O_2 in dark green, without H_2O_2 in light green) for 15 min at 25 °C. (**B**) The volumetric activity of the culture supernatant was determined by oxidation of ABTS for 15 min at 25 °C. (**C**) SDS-PAGE analysis of the 20-fold concentrated fungal culture supernatants. (**D**) Fungal cultures in SG medium during 500 mL shake flask cultivation at 24 °C and 100 rpm on day 6, 9, 12, 15, 19, and 22.

3.4. Enzyme Activity of the C. sublineola Secretome in Different Plant-Based Media

C. sublineola is the causal agent of sorghum anthracnose, one of the most significant diseases in sorghum, which can result in grain yield losses of up to 50%. The hemibiotrophic pathogen infects sorghum at any growth stage, from seedlings to mature plants [55]. The related fungus *C. graminicola* causes economically important anthracnose leaf blight and stalk rot diseases in corn [56]. Buiate et al. showed that *C. sublineola* is also able to colonize non-host corn leaf sheaths after plant cells were killed by localized application of liquid nitrogen [56]. Therefore, a cornmeal-based medium was selected for the next cultivation. Additionally, the effect of lignocellulosic plant material, namely birch sawdust, on enzyme secretion was tested to determine the ligninolytic potential of the fungus. In the presence of easily accessible monosaccharides, fungi tend to prioritize their use over attacking more resistant carbon sources, such as lignin and cellulose, and genes encoding CAZymes are downregulated. This preference is likely influenced by catabolite repression, that regulates

the composition of the secretome in wood-degrading fungi [18,57]. To test this effect on the oxidative activity of *C. sublineola*, glucose was omitted in all media.

Soybean meal without the addition of glucose proved to be an important factor influencing the production and secretion of peroxidases or peroxygenases in agitated liquid culture of *C. sublineola* (Figure 5A,B). The omission of glucose in S medium led to a one-week shift in enzyme activity, resulting in an even higher volumetric activity and a H_2O_2-dependent product yield of 50% oxidized ABTS at day 5, despite an overall two-third lower biomass (Figure A1C). After glucose depletion in SG medium at day 7, enzyme activity as well as pH increased and a maximum product yield of 26% was reached at the 12th cultivation day. Therefore, the exclusion of monosaccharides represents also an effective strategy for enhancing the secretome production of *C. sublineola*.

Furthermore, alteration of the substrate resulted in significant changes in oxidative activity toward ABTS. The substitution of soybean meal for cornmeal resulted in a reduction in enzyme activity of up to 90%. Secretion timing was similar to that in S medium, with a maximum product yield of approximately 8% oxidized ABTS in the presence of H_2O_2 at day 7, except for a brief activity increase at day 20. Notably, *C. sublineola* cultures in cornmeal started to accumulate glucose already at day 3 after inoculation. The nutritional content of a typical corn grain comprises 70–75% starch, 8–10% protein and 4–5% oil [58]. Wood-decay fungi are known to secrete starch-degrading enzymes, such as glucoamylases, which cleave α-1,4- and α-1,6-linkages of starch and produce glucose [59]. Thus, it is likely that *C. sublineola* also possesses starch-degrading capabilities. Visible growth of mycelial agglomerates began around the 10th day, which coincided with the gradual decrease in glucose content in the media (Figure 5D). At the end of the cultivation period, *C. sublineola* reached a similar cell wet weight in cornmeal- and soymeal-based media (Figure A1C). Alkalinization of the culture, which is characteristic for protein degradation, was not detected in the cornmeal-based medium probably due to starch degradation and the relatively low protein concentration of corn grains. It would be interesting to cultivate the fungus for an even longer period in this medium since there was still some glucose left after 26 days. Veratryl alcohol conversion followed a similar trend in all four media, reaching a maximum around day 9 of cultivation, although with varying intensities of activity. Consequently, the trigger mechanism for secretion of veratryl alcohol-oxidizing enzymes was probably not as sensitive for changes in growth substrates as for ABTS-converting enzymes.

C. sublineola also secreted ABTS- and veratryl alcohol-oxidizing enzymes in the lignocellulosic sawdust-based medium. However, cell growth was drastically reduced in this medium (Figure A1C). Comparable to cornmeal medium, product yields of 6% for ABTS and 1–2% for veratryl alcohol were achieved. No growing mycelial aggregate was visible in the liquid, but the medium became more turbid in the course of cultivation (Figure 5D). Low cell growth and pH increase might be due to degradation of the added peptone in the medium. According to Abdel-Rahman et al., birch hardwood is composed of 40% cellulose, 39% hemicellulose and 21% lignin [60]. Since the sawdust used in this study was untreated except for heating for medium sterilization, a pretreatment to reduce particle size and degree of polymerization may be essential for microbial metabolism and growth. The addition of physical methods, like milling and grinding, to achieve particle sizes of less than one millimeter would therefore improve the accessibility of the substrate [61].

In the SDS-PAGE analysis, culture supernatants from all meal-based media revealed distinct bands around 48 kDa and in the range of 60 to 85 kDa, which might be assigned to the eight genome-mined peroxidase- and oxidase-like protein sequences of *C. sublineola* (Figure 5C, Table S2). In contrast, the SDS gel of the BS supernatants showed distinct bands around 38 kDa as well as between 100 and 120 kDa. Consequently, BS medium seemed to induce the expression and secretion of different proteins/enzymes compared to the other media. In this study, the phytopathogen *C. sublineola* was cultivated in different plant-based media for the first time, thereby providing new insights into its growth behavior and

secretome. Further analyses of the underlying enzymes would be an interesting approach for future research.

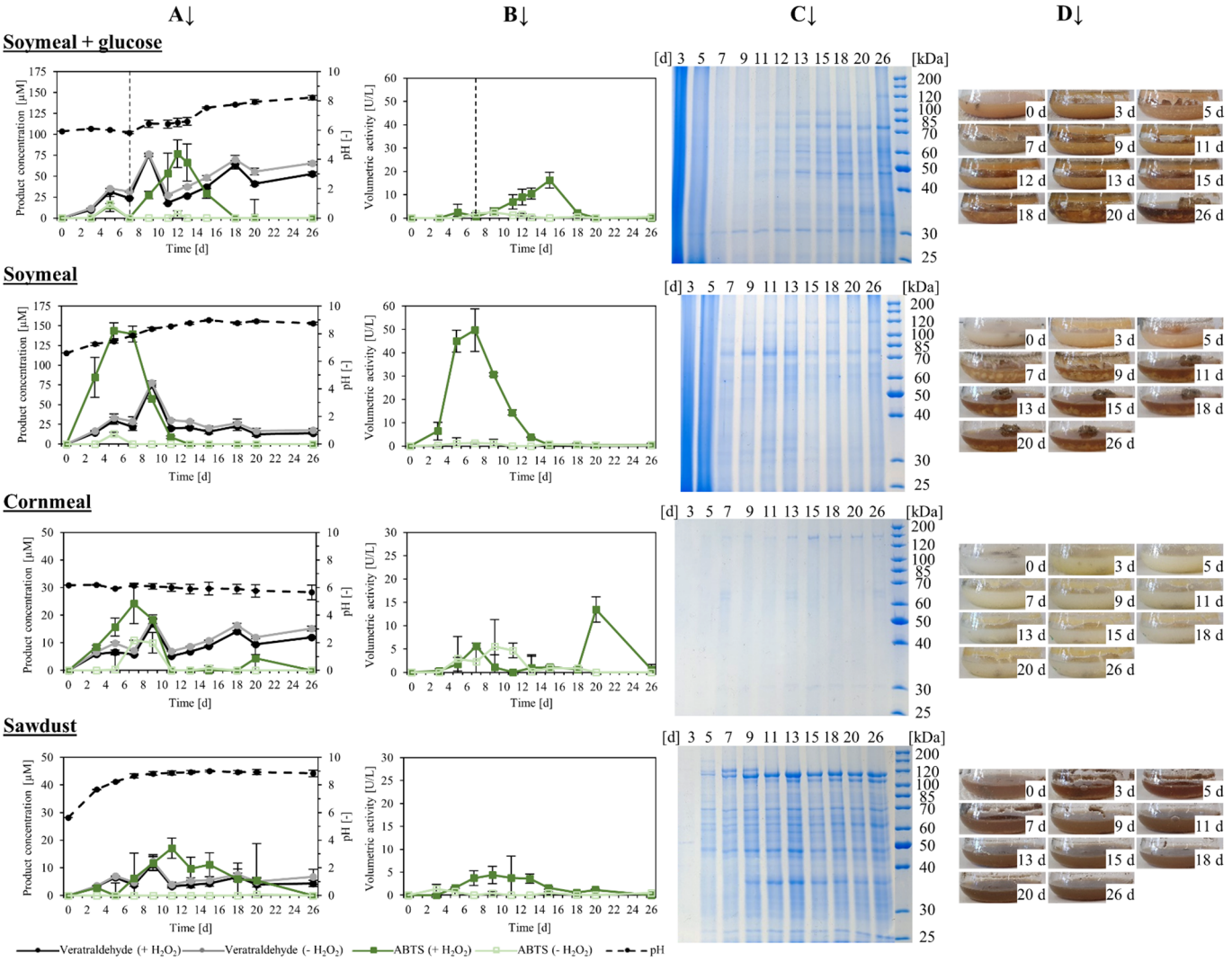

Figure 5. Shake flask cultivation of *C. sublineola* in four complex plant-based media (SG, S, C, and BS medium). Values given are means and average deviations for duplicates. (**A**) pH of the culture supernatant (dashed line) and conversion of 0.5 mM veratryl alcohol to veratraldehyde (circles; with H_2O_2 in black, without H_2O_2 in grey) for 30 min at 21 °C and oxidation of 0.3 mM ABTS (rectangles; with H_2O_2 in dark green, without H_2O_2 in light green) for 15 min at 25 °C. Glucose levels were monitored using glucose test strips. The day of depletion in SG medium is indicated by a thin dashed line. (**B**) The volumetric activity of the culture supernatant was determined by oxidation of ABTS for 15 min at 25 °C. (**C**) SDS-PAGE analysis of the 20-fold concentrated fungal culture supernatants during the 26 days of cultivation. (**D**) Fungal cultures during 1000 mL (SG medium)- or 250 mL (S, C, and BS medium)-shake flask cultivation at 24 °C and 100 rpm after inoculation and on day 3, 5, 7, 9, 11, 12, 13, 15, 18, 20, and 26.

The importance of the type of lignocellulosic substrate for the induction of laccase was demonstrated by Patel and Gupte [62]. They compared various raw materials for the production of laccase by the edible mushroom *Tricholoma giganteum*, including wheat bran, wheat straw, rice bran, and rice straw. Wheat straw was found to be the most suitable material for fungal growth and laccase production in solid-state fermentation, which was improved several-fold, up to 144,000 U per g of dry substrate, under optimized conditions and with the additions of copper sulfate and phenolic inducers. Besides the carbon and

nitrogen source, enzyme production can be affected by the concentration of known inducer compounds, like copper sulfate, *o*-dianisidine, guaiacol, vanillin, pyrocatechol, gallic acid, and veratryl alcohol [62]. Additionally, pH and temperature strongly influence protein secretion, as was shown for the white-rot fungus *Pleurotus ostreatus*. Here, genes, encoding cellulolytic and ligninolytic enzymes, were significantly downregulated when the conditions deviated from 25 °C and an unadjusted pH [63]. Thus, the overall optimization of the cultivation conditions is a promising approach to increase enzyme production and to exploit as well as analyze the whole potential of wild-type *C. sublineola*.

4. Conclusions

Fungi provide a great diversity of oxidative enzymes, especially in their secretome, which are responsible for the decomposition of deadwood or the penetration of living plant cells by pathogens. In order to take full advantage of their biocatalytic potential, it is still essential to identify and investigate wild-type fungi and their secretomes. UniProtKB and similar databases provide several protein sequences for putative heme peroxidases and laccases. However, the determination of the specific functionality of a new enzyme solely based on the protein sequence and comparison is still a challenge. The presented study shows that a genome mining-assisted strain selection combined with an enzyme activity screening enables the identification of the oxidative potential of native fungal secretomes. Thus, this approach can be used as a selection tool for further in-depth analysis, e.g., heterologous expression or proteomics.

Generally, fungi can be utilized as single strains or consortia of microbes to deconstruct lignocellulosic biomass for various industrial applications. However, the induction of protein expression and secretion in fungi is not yet fully understood. Soybean meal without the addition of glucose proved to be a beneficial medium composition, influencing the production and secretion of peroxidases or peroxygenases. Variation in medium composition resulted in clear changes in oxidative activity toward ABTS and in the timing of secretion by the underexploited phytopathogen *C. sublineola*. This basic medium screening demonstrates the importance of substrate choice for enzyme discovery and production in wild-type fungi. Subsequent cultivation experiments with defined media analogous to soybean meal may reveal the essential components crucial for enzyme production. Hence, our results provide new insights into the enzymatic potential of underexploited saprotrophic as well as phytopathogenic fungi, laying the foundation for further investigations and optimization efforts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/appliedchem4030018/s1, Figure S1: LC-MS chromatograms of NBD assay samples; Figure S2: LC-MS chromatograms of veratryl alcohol assay samples; Table S1: Product concentration of veratraldehyde and 4-nitrocatechol; Table S2: Genome-mined putative protein sequences of *Bipolaris victoriae*, *Colletotrichum sublineola*, *Neofusicoccum parvum*, and *Moesziomyces antarcticus*.

Author Contributions: Conceptualization, A.K., K.R. and S.L.; methodology, A.K.; validation, A.K.; formal analysis, A.K.; investigation, A.K.; resources, K.R. and S.L.; data curation, A.K.; writing—original draft preparation, A.K.; writing—review and editing, K.R. and S.L.; visualization, A.K.; supervision, K.R. and S.L.; project administration, K.R. and S.L.; funding acquisition, K.R. and S.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

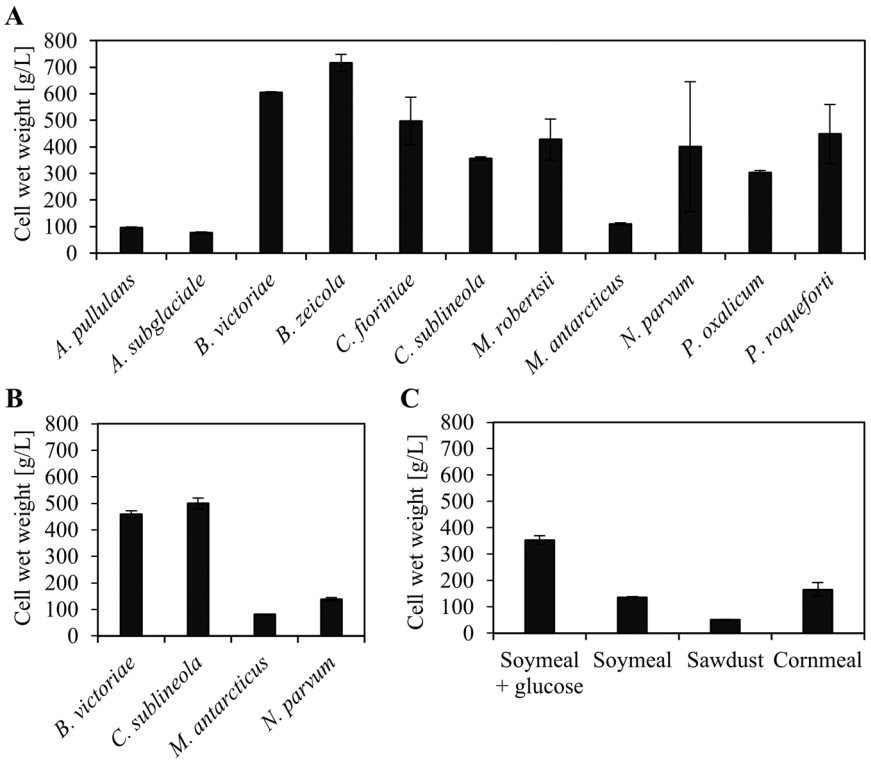

Figure A1. Cell wet weight of fungi cultivated in different plant-based media. (**A**) Cells were harvested after cultivation in SG medium at 24 °C and 100 rpm in 100 mL shake flasks for 28 days. (**B**) Four selected fungi were cultivated again in SG medium at 24 °C and 100 rpm in 500 mL shake flasks for 22 days. (**C**) Cultivation of *C. sublineola* in four complex plant-based media in 1000 mL (SG) or 250 mL shake flasks (S, BS, and C) at 24 °C and 100 rpm for 26 days. Values given are means and average deviations for two replicates.

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