Evaluation of Basidiomycetes Wild Strains Grown in Agro-Industrial Residues for Their Anti-Tyrosinase and Antioxidant Potential and for the Production of Biocatalysts

Anastasia Zerva 1, Nikolaos Tsafantakis 2 and Evangelos Topakas 1,*

1 Industrial Biotechnology & Biocatalysis Group, School of Chemical Engineering, National Technical University of Athens, 9 Iroon Polytechniou Str., Zografou Campus, 15780 Athens, Greece; anazer@chemeng.ntua.gr
2 Department of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, National and Kapodistrian University of Athens, 15771 Athens, Greece; ntsafantakis@pharm.uoa.gr
* Correspondence: vtopakas@chemeng.ntua.gr; Tel.: +30-2107723264

Abstract: White-rot basidiomycetes are the only microorganisms with the ability to produce both hydrolytic (cellulases and hemicellulases) and oxidative (ligninolytic) enzymes for degrading cellulose/hemicellulose and lignin. In addition, they produce biologically active natural products with important application in cosmetic formulations, either as pure compounds or as standardized extracts. In the present work, three wild strains of Basidiomycetes fungi (Pleurotus citrinopileatus, Abortiporus biennis and Ganoderma resinaceum) from Greek habitats were grown in agro-industrial residues (oil mill wastewater, and corn cob) and evaluated for their anti-tyrosinase and antioxidant activity and for the production of biotechnologically relevant enzymes. P. citrinopileatus showed the most interesting tyrosinase inhibitory activity, while A. biennis showed the highest DPPH(2,2-diphenyl-1-picryl-hydrazyl) scavenging potential. Corn cobs were the most appropriate carbon source for maximizing the inhibitory effect of fungal biomasses on both activities, while the use of oil mill wastewater selectively increased the anti-tyrosinase potential of P. citrinopileatus culture filtrate. All strains were found to be preferential lignin degraders, similarly to most white-rot fungi. Bioinformatic analyses were performed on the proteome of the strains P. citrinopileatus and A. biennis, focusing on CAZymes with biotechnological relevance, and the results were compared with the enzyme activities of culture supernatants. Overall, all three strains showed strong production of oxidative enzymes for biomass conversion applications.

Keywords: basidiomycetes; novel enzymes; biocatalysts; anti-tyrosinase activity; antioxidant activity; laccases; LPMOs

1. Introduction

White-rot basidiomycetes are the only microorganisms able to produce hydrolytic (cellulases and hemicellulases) and oxidative (cellulases, ligninases) enzymes for attacking both cellulose/hemicellulose and lignin [1]. Ligninolytic enzymes can be applied in numerous fields of industry, e.g., chemical, fuel, food, agriculture, paper, textile and cosmetics industries. Lignin is highly recalcitrant to microbial degradation and, together with hemicellulose, creates a complex structure that inhibits the degradation/hydrolisis of cellulose in plant cells [2]. Certain white-rot basidiomycetes in particular, are able to degrade lignin, cellulose and hemicellulose concomitantly, while others attack lignin selectively by secreting enzymes, collectively named “ligninases” [3]. Ligninases can be divided into separate classes, namely, phenol oxidases (laccases) and heme peroxidases [lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP)]. Apart from ligninolytic enzymes, white-rot fungi also secrete cellulases, xylanases and other hemicellulases. These enzymes act on long-chain polysaccharides, mainly cellulose...
and hemicellulose, resulting in the hydrolysis of these materials to their monomer sugars, of six and five carbon atoms, respectively [4]. The substrates used for fungal growth are usually agricultural or agro-industrial residues of low economic value and, in some cases with toxic effects to the environment (e.g., olive-mill wastes). In Greece, crop by-products (e.g., straw) as well as olive mills (e.g., leaves, wastewater and sludge-like effluents) and wineries (e.g., grape marc) wastes present significant problems in their processing and/or disposal. Especially for olive oil, the world production for the 2019/2020 season amounts to 3,207,000 t, of which 275,000 t (8.6%) is produced in Greece, amounting to a 48.6% increase compared to the previous years, according to the International Olive Council [5]. This results in a similarly increasing trend in the production of related wastes.

Recent studies revealed that the composition of cultivation substrates can significantly affect the growth, nutritional and biological properties of mushrooms [6,7]. However, our knowledge regarding the effect that cultivation substrates could exert on the production of ligninolytic enzymes by edible/medicinal basidiomycetes is still limited.

White-rot basidiomycetes include several edible/medicinal mushrooms of significant nutraceutical value and they are known to produce secondary metabolites with important applications in cosmetic formulations [8–10]. Secondary metabolites are low-molecular-weight compounds (MW < 1500), with considerable structural and functional complexity. In the cosmetic sector, they can be found in several antiaging formulations due to their antioxidant and anti-tyrosinase properties. Oxidative stress is one of the prevailing causes of skin aging [11]. Many reports have demonstrated the ability of microorganisms to biosynthesize antioxidant secondary metabolites and contribute to the maintenance of a balanced equilibrium among oxidants and antioxidants. Phenolic compounds and carotenoids are among the most representative classes of natural products with significant antioxidant properties [12]. Tyrosinase is the key enzyme of melanin biosynthesis in microorganisms, plants, and animals. It plays an important role in the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and in the oxidation of L-DOPA to dopaquinone [13]. Tyrosinase inhibitors are mainly used for the treatment and prevention of hyperpigmentation in the epidermal layers of the human skin. For this reason, tyrosinase inhibitors are also known as skin whitening agents. Uneven pigmentation is one of the major changes associated with aging. It may lead to blotches, patches of brown to grey discoloration or freckling. Most of the tyrosinase inhibitors derive from natural sources including various plants and microorganisms (terrestrial or marine). Among them, kojic acid, azelaic acid and ellagic acid are the most common skin whitening agents [14]. Despite the increase in clinical and industrial demand for tyrosinase inhibitors, nowadays there are very few compounds certified for clinical use [15]. Thus, there is a strong need for new anti-tyrosinase agents.

Towards the potential exploitation of white-rot basidiomycetes grown in agro-industrial residues in the cosmetic and biocatalysis industries, wild strains of edible/medicinal saprotrophic basidiomycetes isolated from diverse habitats of Greek territory were evaluated for their antiaging potential (anti-tyrosinase and antioxidant activity) and for the production of biotechnologically relevant enzymes with cellulolytic and ligninolytic activity. The present study focused on the description of new wild Basidiomycetes strains as alternatives for agro-industrial residue valorization, aiming at the exploitation of both the fungal biomass for the extraction of bioactive compounds, and the spent liquid substrate as a source rich in industrially relevant enzymes, in the context of the circular economy.

2. Results and Discussion
2.1. Anti-Oxidant and Anti-Tyrosinase Activity of Mushroom Extracts

Towards the potential exploitation of wild strains of edible/medicinal basidiomycetes in cosmetic and biocatalysis industries, liquid cultures of Abortiporus biennis, Pleurotus citrinopileatus and Ganoderma resinaceum were prepared, using different lignocellulosic waste materials as carbon sources. The fungal cultures were evaluated for their antioxidant
and anti-tyrosinase activity and for their potential production of enzymes with cellulolytic and ligninolytic activity, respectively.

For all strains, CC was the most appropriate carbon source for maximizing the anti-aging potential of fungal mycelia (Figures 1 and 2). Among the biomass extracts, \textit{P. citrinopileatus} showed the most interesting tyrosinase inhibitory activity (32.00 ± 0.92\%), followed by \textit{G. resinaceum} (27.25 ± 0.96\%) and by \textit{A. biennis} (25.20 ± 1.85\%) (Figure 1a). It is worth noting here that the use of olive-oil mill wastewater (OMWW) as carbon source had no significant effect on the anti-tyrosinase potential of the EtOAc extracts obtained from the biomasses of \textit{G. resinaceum} and of \textit{A. biennis}, while for \textit{P. citrinopileatus}, a complete loss of bioactivity was observed (Figure 1a). Regarding biomass hydroalcoholic extracts, which were significantly less active, OMWW leads to a further reduction in the anti-tyrosinase potential. Following this concept, the DPPH(2,2-diphenyl-1-picryl-hydrazyl) scavenging potential of the generated extracts significantly decreased when the OMWW was used as carbon source (Figure 2a). Among the supernatants, the anti-tyrosinase potential of \textit{P. citrinopileatus} culture filtrate showed the most promising activity, since the OMWW-EtOAc-LL extract exerted a 36.96 ± 1.25\% enzyme inhibition (Figure 1b). Taking the absence of any relevant anti-tyrosinase activity of the OMWW growth medium (−16.59 ± 3.27) into consideration, the OMWW-EtOAc-LL extract of \textit{P. citrinopileatus} represents the most interest extract for future chemical investigation (Figure 1b). With the exception of \textit{P. citrinopileatus}, the use of CC as a carbon source was apparently proven to be the most appropriate for maximizing the anti-tyrosinase potential of culture supernatants (Figure 1b). However, the anti-tyrosinase activity expressed by the CC-EtOAc-LL extract originated from the growth medium without fungi (41.82 ± 1.44\%) allowed us to exclude \textit{A. biennis} and to assign a slight increment of the skin whitening effect, in the form of tyrosinase inhibition (\textit{p} < 0.01) exclusively to \textit{G. resinaceum} CC (Figure 1b). In contrast to the anti-tyrosinase potential of the EtOAc-LL extracts, supernatants failed to reproduce any significant scavenging effect against the DPPH radical at 200 µg mL⁻¹ (Figure 2b). These data suggest CC is the more appropriate carbon source for gaining maximum antioxidant and anti-tyrosinase activity from the aforementioned biomasses, while the OMWW cultivation media was the most appropriate for maximizing the anti-tyrosinase potential of \textit{P. citrinopileatus} culture supernatant.

Figure 1. Tyrosinase inhibitory activity of fungal cultures (300 µg mL⁻¹). (a) Biomasses; (b) Culture supernatants; Positive control: Kojic Acid (2 µg/mL), inhibition\% = 44.40 ± 1.37; inhibition\% of non-inoculated growth media: CC-EtOAc-LL = 41.82 ± 1.44, OMWW-EtOAc-LL = −16.59 ± 3.27, CC-H₂O = −5.05 ± 3.07, OMWW-H₂O= −7.91 ± 1.95. Experiments were performed in triplicate; Error bars indicate ± standard errors; (\textit{p} < 0.001, HSD Tukey).
To the best of our knowledge, the anti-tyrosinase potential of *P. citrinopileatus* has been only reported from fruiting bodies of wild mushrooms [16,17]. Interestingly, our findings were in agreement with those reported by Alam et al. [16] and significantly higher than those of Shu et al. [17], showing a lower potential to prevent the oxidation of L-DOPA. Therefore, liquid cultures of this popular and edible mushroom using CC agricultural waste as a carbon source could equally be exploited for the production of bioactive extracts with skin whitening properties and, at the same time, contribute to the effective waste disposal of agro-residues, as previously reported by several authors for different *Pleurotus* species [18–20]. Similar to the anti-tyrosinase potential, the use of CC agricultural waste as a carbon source offered the highest scavenging effect against the DPPH radical. However, when compared to the literature data related to the antioxidant potential of fruiting bodies, the antiradical potential was significantly reduced [17,21,22]. This may be explained by the different composition of biologically active substances between the mycelia obtained from submerged cultures and the fruiting bodies obtained by solid-state cultivation [23]. Our findings were mostly in agreement with those reported previously [24,25], showing a moderate to low DPPH scavenging activity of *P. citrinopileatus* mycelium when compared to other *Pleurotus* species.

The highest DPPH scavenging activity was shown from *A. biennis* using the CC agricultural residue. As in the case of *P. citrinopileatus*, the use of OMWW failed to induce any antioxidant activity. Macáková et al., [26] showed a low DPPH scavenging activity for *A. biennis* among 30 different mushroom species (fruiting bodies). These data indicate that the cultivation conditions can have a great impact on metabolite production for this fungus. The use of *A. biennis* to treat OMWW, a major cause of environmental pollution in olive-oil producing regions, was firstly proposed from Aggelis et al. [27] and Koutrotsios and Zervakis [28]. The authors showed that *A. biennis* was the best-performing strain for the reduction of the total phenolic content. However, we demonstrated that the generated extracts of this fungus, cultivated using OMWW agricultural residue, lost their scavenging ability, while exhibiting a decreased whitening effect. To the best of our knowledge, there are no available data correlating the production of secondary metabolite with the antioxidant and skin whitening activities of this species.

Several studies related to fruiting bodies of *G. resinaceum* have reported considerable free radical scavenging activity, strongly correlating to their total phenolic content, to the presence of characteristic triterpenoids and to antioxidative exopolysaccharides [29–31].
A noticeable whitening effect was also shown by Zengin et al., [29]. Similar to the case of \textit{P. citrinopileatus} and \textit{A. biennis}, the use of OMWW agricultural waste failed to induce any antioxidant potential. Our results related to the DPPH scavenging activity of \textit{G. resinaceum} cultivated in the presence of CC agricultural waste were mostly in agreement with those reported from Saltarelli et al. [32], showing a moderate antioxidant activity of mycelial cultures grown in an optimized liquid medium. Apigenin, benzoic acid, catechin and epicatechin represented the major compounds [32]. However, El-Katony et al. [33] emphasized that the scavenging ability and the production of secondary metabolites show significantly different trends on solid-state cultures with different substrates (PDA and orange peel). Our findings suggest that \textit{G. resinaceum} could be successfully cultivated using specific agro-waste residues, contribute to the biodegradation process and, in relation to the cultivation substrate, be exploited for the production of bioactive extracts. In that regard, is worth mentioning that \textit{G. resinaceum} was successfully applied in the biodegradation of reactive textile dyes [34].

2.2. Biocatalyst Production in Different Induction Media

The first step towards exploring the enzymatic variability of the studied strains in regard to lignocellulose degradation was to assemble the predicted CAZymes of the available genome sequences using state-of the-art annotation tools, such as dbCAN2 meta server. The annotation was performed for the publicly available genome sequences of \textit{P. citrinopileatus} and \textit{A. biennis}. The results are shown in Table 1.

| Specific Activity                         | CaZy Families | \textit{A. biennis} | \textit{P. citrinopileatus} |
|------------------------------------------|---------------|---------------------|-----------------------------|
| Cellulases                               | Endoglucanases| GH 5, 7, 12, 45     | 14                          | 17                          |
|                                          | Cellobiohydrolases | GH 6, 7         | 4                            | 10                          |
|                                          | β-glucosidases  | GH 1, 3           | 2                            | 5                           |
| Xylanases                                | Endoxylanases  | GH 10, 11         | 6                            | 15                          |
|                                          | β-xylosidases  | GH 3, 43          | 4                            | 7                           |
| Oxidases                                 | Laccases       | AA 1               | 8                            | 16                          |
|                                          | Peroxidases    | AA 2               | 12                           | 11                          |
|                                          | Alcohol oxidases and cellobiose dehydrogenases | AA 3 | 3 | 16 |
|                                          | Lytic polysaccharide monoxygenases | AA 9, 10, 11, 13, 14 | 12 | 28 |

As shown in Table 1, the genomes of both strains include all the necessary enzymatic activities for the breakdown of major lignocellulose components, and, in most cases, in multiple gene copies. \textit{P. citrinopileatus} was found to contain more copies of oxidative enzymes than \textit{A. biennis}, as well as more endoxylanase copies. Especially for laccases, \textit{Pleurotus} species are known to be potent producers [35], and \textit{P. citrinopileatus} was shown to be no exception. Nonetheless, both strains are shown to possess the biocatalytic potential for complete degradation of lignocellulosic biomass and relative substrates. However, the existence of the required genes does not necessarily lead to efficient induction, expression, and secretion of the respective proteins. For this reason, the aforementioned fungal strains, together with \textit{G. resinaceum}, which is a strain with an unknown genome sequence, were grown in different lignocellulosic materials as carbon sources, and the profile of secreted enzyme activities was explored in reaction supernatants.

OMWW is the liquid by-product of olive oil production process by the three-phase decanter system, which is widely popular in Mediterranean countries. The dark color, strong odor and high phenolic load of OMWW pose significant problems in its disposal in landfills and biological treatment plants; therefore, detoxification is required before its environmental release [6,7]. White-rot basidiomycetes have been extensively used for the biological treatment of such effluents, due to their potent oxidative enzyme systems [6,7]. All of the studied strains were found able to achieve complete breakdown of OMWW phenolic load, as shown in Figure 3. The maximum phenol degradation was observed by...
P. citrinopileatus, reaching up to 94.3 ± 0.9% phenol degradation and up to 86.1 ± 0.6% color removal.

Figure 3. OMWW degradation during growth of P. citrinopileatus (black circles), A. biennis (white circles) and G. resinaceum (black inverted triangles), on OMWW. (a) Removal of phenols, (b) decolorization.

The time profile of laccase production is shown in Figure 4a. Surprisingly, P. citrinopileatus was found to be the least potent laccase producer from the three studied strains, while G. resinaceum showed the highest laccase production, up to 2014.93 ± 86.9 U L$^{-1}$.

Regarding peroxidase activity (Figure 4b), G. resinaceum was also found to produce the highest activity, up to 92.85 ± 2.3 U L$^{-1}$.

Figure 4. Laccase activity (a) and peroxidase activity (b) during the growth of P. citrinopileatus (black circles), A. biennis (white circles) and G. resinaceum (black inverted triangles), on OMWW.

The next step was to study the enzyme production during growth on an untreated lignocellulosic substrate, therefore, the three fungal strains were grown in CC, in shaking liquid cultures. Endocellulase, endoxylanase, and laccase activities were monitored during the course of the fermentation. The results for laccase and peroxidase activities are shown in Figure 5. Laccase and peroxidase activities for all strains was found to be significantly lower than when grown on OMWW, which is expected due to the low lignin content of the substrate. Again, G. resinaceum was found to be the most potent laccase producer from the three strains. A. biennis was shown to produce higher peroxidase activities than the other strains, but similar activity to the OMWW cultures. Surprisingly, endocellulase and endoxylanase activities were not detected at any timepoint during the course of the fungal cultures. This result might be partly explained by the physiology of white-rot basidiomycetes, most of which are preferential lignin degraders [3].
Despite the absence of detectable endocellulase or endoxylanase activity, soluble reducing sugars were detected in the culture supernatants. All strains were found to produce soluble sugars during the course of the reaction, up to 0.66 mg mL\(^{-1}\) ± 0.004 for \textit{P. citrinopileatus}, 0.59 ± 0.04 mg mL\(^{-1}\) for \textit{A. biennis} and 0.41 ± 0.01 mg mL\(^{-1}\) for \textit{G. resinaceum}, compared to the concentration of the control (0.33 ± 0.003 mg mL\(^{-1}\)), indicating the presence of at least some enzyme activity on biomass polysaccharides. All the above data, together with the existence of several lytic polysaccharide monoxygenases (LPMO) gene copies in the genomes of \textit{P. citrinopileatus} and \textit{A. biennis}, support the existence of potent LPMO activity for the efficient degradation of polysaccharides.

In order to detect LPMO activity, the three fungal strains were grown in microcrystalline cellulose as sole carbon source. After 15 days of growth, the supernatants were analyzed for LPMO activity, and the results are shown in Figure 6. As shown, LPMO activity was detected in the supernatants of all fungal strains, albeit the presence of oxidized sugars [36] is more prominent in \textit{P. citrinopileatus} samples. Our results confirm the expression of LPMOs from the studied strains for cellulose degradation; however, the exact role and significance of LPMO activity during fungal growth in natural complex substrates is still unknown.
graders [37,38], consuming first the lignin part of biomass, by the concerted expression of oxidizing enzymes. *G. lucidum* in particular, although it contains a wide array of cellulase and xylanase genes, was found to secrete mostly lignin-degrading enzymes in the mycelium phase [38], while polysaccharide-acting enzymes were secreted mainly during fruiting body formation. These results may explain the absence of cellulase and xylanase activities in the supernatant of liquid cultures in the present work. As regards the data from *P. ostreatus* secretomes, the most abundant lignocellulose-degrading enzymes produced belonged to accessory hydrolases, such as CaZymes GH1, GH3 and CE16, and not those acting on the polysaccharide backbone [37].

Regarding LPMO activity, although *P. ostreatus* genome contains 18 respective genes, no LPMO was detected in the secretome of the fungus under inducing conditions [37]. This is in contrast with the present study, where LPMO activity was detected in *P. citrinopileatus* supernatants. On the contrary, AA9 LPMOs were found to be upregulated in inducing conditions, during fruiting body formation, together with cellulose-acting enzymes, in the secretome of *G. lucidum* [38].

3. Methods

3.1. Microorganisms and Culture Procedures

The *P. citrinopileatus* LGAM 28684, *A. biennis* LGAM 436 and *G. resinaceum* LGAM 334 strains used for this study, were obtained from the fungal culture collection of the Laboratory of General and Agricultural Microbiology (Agricultural University of Athens, Athens, Greece). The strains were maintained in Potato Dextrose Agar plates (PDA-AppliChem GmbH, Darmstadt, Germany) at 4 °C. Agar plugs (6 mm diameter) were used as inocula for the liquid cultures. OMWW was obtained from an olive oil mill with a three-phase decanter in Kalamata (Peloponnese, S.W. Greece, 14 March 2018) and maintained at −20 °C. The composition and physicochemical properties of OMWW were previously assessed [39]. Prior to use, pH was adjusted to 6 with 3N NaOH, and the suspended solids were removed. For the preparation of liquid cultures, OMWW was diluted at a final concentration of 50% (v/v) with 100 mM phosphate buffer pH 6 to a final volume of 50 mL. Alternatively, CC or Avicel were added at a final concentration of 30 g L⁻¹ as a carbon source. Corn steep liquor was added as a nitrogen source at a final concentration of 3 g L⁻¹. After inoculation, liquid cultures were incubated at 26 °C, 100 rpm, unless otherwise stated. Samples were taken at selected time intervals and centrifuged (3000 rpm, 10 min), and then the supernatant was used for analysis and determination of enzyme activities, as described in the following paragraphs.

3.2. Extraction of Fungal Cultures

For the evaluation of the antioxidant and tyrosinase inhibitory activity, biomasses were separated from culture supernatants by filtration using Whatman filter paper No 4. Biomasses were first lyophilized and then subjected to a sequential ultrasound-assisted extraction with an Elmasonic S100H unit (Elma Schmidbauer GmbH, Singen, Germany) using Ethyl Acetate (EtOAc) and 50% Methanol/Water (MeOH/H₂O). 0.4 g of dried biomass was extracted at room temperature for 40 min using 10 mL of solvent per cycle. Each sample was extracted three times, and supernatants were centrifuged (4000 rpm, 3 min), combined and dried under reduced pressure using a centrifugal vacuum concentrator (Genevac Ltd., Suffolk, UK). Supernatants were subjected to a Liquid-Liquid extraction with EtOAc. 20 mL of each supernatant was extracted three times with 20 mL of EtOAc and the upper phases were combined and dried under reduced pressure using a centrifugal vacuum concentrator. The remaining aqueous part was lyophilized. In total, each fungal culture offered four different extracts: two related to the biomass (EtOAc and 50% MeOH/H₂O) and two related to the supernatants (EtOAc-LL and H₂O). Dried extracts were immediately stored at −20 °C prior to analysis.
3.3. Free Radical Scavenging (DPPH)

This method is based on the reduction in the free radical DPPH (2,2-diphenyl-1-picryl-hydrazyl) by antioxidant agents, which leads to the discoloration of the DPPH solution; the color change is monitored spectrophotometrically using the TECAN Infinite M200 Pro microplate reader employing the Magellan™ software (Tecan Group, Männedorf, Switzerland). A protocol based on Lee et al. [40] was scaled down for application in a 96-well plate reader. A stock solution of 0.314 mM DPPH in absolute ethanol was prepared and kept in the dark at ambient temperature. Stock solutions of samples in DMSO (4 mg/mL) were prepared and dilutions were made in the testing concentration in the same solvent. In 96-well plates, 10 µL of extract in DMSO and 190 µL of DPPH solution were mixed and incubated for 30 min at ambient temperature in dark. Absorbance was measured at 517 nm in a TECAN microplate reader. Blanks for every sample w/o DPPH were also performed. Gallic acid was used as a positive control. The percentage DPPH scavenging was estimated by the following equation: \(((A-B) - (C-D))/((A-B) \times 100)\), where A: Control (w/o sample), B: Blank (w/o sample, w/o DPPH), C: sample, D: Blank sample (w/o DPPH). Extracts were initially tested at 200 µg/mL (final concentration).

3.4. Tyrosinase Assay–Estimation of Skin Whitening Activity

The ability of the extracts to inhibit the oxidation of L-DOPA (L-3,4-dihydroxyphenylalanine) to dopaquinone and subsequently to dopachrome by the enzyme tyrosinase was evaluated, as previously described [41]. Extracts were dissolved in DMSO to stock solutions of 10 mg/mL and were diluted in the proper concentration in phosphate buffer 1/15 M (NaH$_2$PO$_4$/Na$_2$HPO$_4$), pH 6.8. Final concentrations of DMSO in the well did not exceed 3%. In 96-well plates, 80 µL of phosphate buffer 1/15 M (NaH$_2$PO$_4$/Na$_2$HPO$_4$), pH 6.8, 40 µL of sample in the same buffer and 40 µL mushroom tyrosinase (92 Units/mL) in the same buffer, were mixed. The contents of each well were incubated for 10 min at 25 °C, before 40 µL of 2.5 mM L-DOPA in the same buffer were added. After incubation at 25 °C for 5 min, the absorbance at 475 nm of each well was measured using a TECAN plate reader. Blanks for every sample w/o tyrosinase were also performed. Kojic acid was used as positive control. The percentage inhibition of the tyrosinase activity was calculated by the following equation: \((A - B) - (C - D))/((A - B) \times 100)\), where A: Control (w/o sample), B: Blank (w/o sample, w/o tyrosinase), C: Sample, D: Blank sample (w/o tyrosinase). Extracts and fractions were tested at 300 µg/mL (final concentration in the well).

3.5. Enzyme Assays

Laccase (E.C. 1.10.3.2: benzenediol: oxygen oxidoreductase) activity was determined at 420 nm, monitoring the oxidation of 2 mM ABTS (2,2- amino bis(3-ethylbenzothiazoline-6-sulphonic acid, $\varepsilon$$_{420} = 36,000$ M$^{-1}$ cm$^{-1}$) in 100 mM citrate-phosphate buffer pH 4 and 30 °C for 10 min. One Unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of product per min, in the above conditions. Peroxidase activities were measured as previously described [42], with 2.5 mM DMAB (3-dimethylaminobenzoic acid), 0.05 mM MBTH (3-methyl-2-benzo-thiazoline hydrazone) in the presence of 0.05 mM H$_2$O$_2$, in succinic acid-sodium lactate buffer 0.1 M pH 4.5. Color change was monitored for 3 min in 25 °C at 590 nm ($\varepsilon$$_{590nm} = 32,900$ M$^{-1}$ cm$^{-1}$). Cellulase and xylanase activities were measured as previously described [43], using carboxymethyl cellulose, Avicel or birchwood xylan as substrates. The determination of reducing sugars was performed with the 3,5-dinitrosalicylic acid (DNS) method [44]. For the detection of LPMO activity, 200 µL of culture supernatant were added to 500 µL of phosphoric acid-swollen cellulose (PASC) 2% (w/v) in 50 mM acetate buffer pH 5.2. Cysteine was added as electron donor at a final concentration of 1 mM. The mixture was incubated for 16 h in 45 °C and 1000 rpm. The reaction was terminated by boiling, and the supernatant was analyzed for oxidized sugars with HPAEC-PAD (High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection) as described previously [45].
3.6. Determination of OMWW Total Phenolic Content and Decolorization

Total phenol content was determined as previously described [42], and it was expressed in ppm of gallic acid equivalents. OMWW decolorization was calculated by measuring the absorbance at 525 nm, as previously described [42].

3.7. Bioinformatics Analyses

Automated CAZyme annotation was performed to the predicted protein sequences of *P. citrinopileatus* and *A. biennis*, with the dbCAN2 meta server, employing the HMMER, DIAMOND and HotPep tools [46].

3.8. Statistical Analysis

Data analyses and statistics were performed using GraphPad Prism 6.0 and the open-source software RStudio version 1.2.5033 (2009–2019 RStudio, Inc). The ANOVA and Tukey’s test were carried out with the following package: “agricolae” [47]. Statistical significance is indicated in graphs by letters.

4. Conclusions

In the present work, a screening study was performed for three native white-rot basidiomycete strains, *P. citrinopileatus*, *A. biennis* and *G. resinaceum* in two different agro-industrial byproducts: OMWW and CC. *P. citrinopileatus* showed the most interesting tyrosinase inhibitory activity while *A. biennis* showed the highest DPPH scavenging potential. For all strains, CC was the most appropriate carbon source for maximizing the inhibitory potential of fungal biomasses on both activities, while the use of OMWW selectively increased the anti-tyrosinase effect of *P. citrinopileatus* culture filtrate. All three strains showed significant degradative potential, by removing more than 80% of OMWW phenols, and by the production of high titers of laccase activity. The three strains were found to be preferential lignin degraders, similarly to most white-rot fungi. Automated annotation tools revealed that *P. citrinopileatus* and *A. biennis* contain multiple copies of all the genes necessary for complete lignocellulose breakdown. Overall, all three strains show promising anti-tyrosinase and antioxidant properties and significant potential for biodegradation applications, but also for the discovery of novel enzyme activities.

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**Data Availability Statement:** The data presented in this study are available in the article.

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