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Greek Basidiomycete Wild Strains for the Production of Bioactive Compounds and Enzymes with Applications in Cosmetic and Biocatalysis Industries †

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Abstract: White-rot basidiomycetes are the only microorganisms able to produce both hydrolytic (cellulases and hemicellulases) and oxidative (ligninolytic) enzymes for attacking (hemi)cellulose and lignin, while they also produce secondary metabolites with important applications as cosmeceuticals. In the present work, three wild strains of Basidiomycete fungi (*Pleurotus citrinopileatus*, *Abortiporus biennis* and *Ganoderma lucidum*) were grown in agroindustrial residues, such as olive oil mill wastewater and corn cob. The cultures were examined in regard to the production of biotechnologically relevant enzymes and bioactive compounds. All strains were found to be preferential lignin degraders. Bioinformatic analyses were performed on the putative proteome of the strains *P. citrinopileatus* and *A. biennis*, focusing on CAZymes with biotechnological relevance, and the results were compared to the data from the enzyme activities in the culture supernatants. The antiaging proprieties of fungal biomasses have been evaluated in relation to the different culture media. *P. citrinopileatus* showed the highest skin whitening activity, while *A. biennis* the highest DPPH scavenging potential. For all strains, corn cobs was the most appropriate carbon source for the production of bioactive small molecules with anti-aging activity. Overall, all strains showed promising antiaging properties and strong production of promising oxidative enzymes for biomass conversion applications.

Keywords: mushrooms; basidiomycetes; novel enzymes; biocatalysts; natural bioactive compounds; secondary metabolites; laccases; LPMOs

1. Introduction

White-rot basidiomycetes are the only microorganisms producing hydrolytic (cellulases and hemicellulases) and oxidative (ligninolytic) enzymes for attacking both cellulose/hemicellulose and lignin. In addition, they produce secondary metabolites with important applications in the cosmetics industry. Especially ligninolytic enzymes can be applied in numerous fields of industry, e.g. fuel, food, agriculture, paper, textile and cosmetics industries. Lignin is highly recalcitrant to microbial degradation [1]. 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" [2]. Ligninases can be divided in separate classes, namely phenol oxidases (laccases) and heme peroxidases. 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. The substrates used for fungal growth are usually agricultural or agro-industrial residues of low economic value and in many cases with toxic effects (e.g. olive-mill wastes). Recent studies revealed that the composition of cultivation substrates can significantly affect nutritional properties of mushrooms [3] or their antioxidant activity. Unfortunately, a huge gap still exists in our knowledge regarding the effect that cultivation substrates could exert on the production of bioactive metabolites, and/or ligninolytic enzymes by edible/medicinal basidiomycetes.

In the cosmetic sector, secondary metabolites can be found in several antiaging formulations due to their anti-oxidant and anti-tyrosinase proprieties. Most of tyrosinase inhibitors derive from natural sources including various microorganisms [4]. Tyrosinase is the key enzyme of melanin biosynthesis in microorganisms, plants, and animals. Tyrosinase inhibitors are mainly used for the treatment and prevention of hyperpigmentation in the epidermal layers of the human skin. Despite that the clinical and industrial demands for tyrosinase inhibitors increase, nowdays there are very few compounds certified for clinical use [5]. Thus, there is a strong need for new anti-tyrosinase agents.

In the present work, wild strains of edible/medicinal basidiomycetes isolated from diverse habitats of Greek territory were evaluated for the production of enzymes with cellulolytic and ligninolytic activity and bioactive small molecules with anti-aging activity.

2. Methods

2.1. Microorganisms and Culture Procedures

The *P. citrinopileatus* LGAM 28684, *Abortiporus biennis* LGAM 436 and *Ganoderma 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). OMWW was obtained from an olive oil mill with a three-phase decanter in Kalamata (Peloponnese, S.W. Greece) and maintained at −20 °C [6]. 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, corn cobs (CC) or Avicel were added at a final concentration of 30 g L^{-1} as carbon source. Corn steep liquor was added as 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, centrifuged (3000 rpm, 10 min), and the supernatant was used for analysis and determination of enzyme activities.

For the evaluation of the antioxidant and skin whitening activity, biomasses were separated from culture supernatants by filtration using whatman filter paper No 4 and lyophilized. 0.4 g of each dried biomass was subjected to ultrasound-assisted extraction for 40 min at 25 °C using 10 mL of ethyl acetate. Each sample was extracted 3 times, supernatants were centrifuged (4000 rpm, 3 min), combined and dried under reduced pressure using a centrifugal vacuum concentrator. Extracts were immediately stored at −20 °C prior to analysis.

2.2. Enzyme Assays and Analyses

Laccase activity was determined as described in [7]. Cellulase and xylanase activities were measured as described in [8], using carboxymethyl cellulose, avicel or birchwood xylan as substrates. The determination of reducing sugars was performed with the DNS method [9]. 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, as described previously [10].

Total phenols content was determined as previously described [7] and it was expressed in ppm of gallic acid equivalents. OMWW decolorization was calculated by measuring the absorbance at 525 nm, as previously described [7].

2.3. 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 [11].

2.4. Free Radical Scavenging (DPPH) and Tyrosinase Inhibition Assay

The protocol based on [12] was used as follows: A stock solution of 0.314 mM DPPH in ethanol was prepared. Stock solutions of samples in DMSO (4 mg mL^{-1}) were prepared. 10 μl of extract in DMSO and 190 μl of DPPH solution were mixed and incubated for 30 min at 25 °C in the dark. Absorbance was measured at 517 nm. Gallic acid was used as positive control. The percentage DPPH scavenging was estimated by the following equation: [(A − B) − (C − D)] / (A − B) × 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 tested at 200 μg mL^{-1} and 100 μg mL^{-1} (final concentration).

The anti-tyrosinase ability of the extracts was evaluated as described in [13]. Extracts were dissolved in DMSO and diluted in phosphate buffer 1/15 M, pH 6.8. In 96-well plates, 80 μl of phosphate buffer 1/15 M, pH 6.8, 40 μl of sample and 40 μl mushroom tyrosinase (92 Units mL^{-1}) were mixed. The contents of each well were incubated for 10 min at 25 °C, before 40 μl of 2.5 mM L-DOPA were added. After incubation at 25 °C for 5 min, the absorbance at 475 nm was measured using a TECAN plate reader. 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) × 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^{-1} and 100 μg mL^{-1} (final concentration in the well).

3. Results and Discussion

In our first steps towards the potential exploitation of wild strains of edible/medicinal basidiomycetes in cosmetic and biocatalysis industries, we prepared liquid cultures of A. biennis, P. citrinopileatus and G. resinaceum using different lignocellulosic waste materials as carbon sources. The fungal biomasses were extracted and evaluated for their anti-oxidant and anti-tyrosinase acitivities while culture supernatants were evaluated for the production of enzymes with cellulolytic and ligninolytic activity, respectively. P. citrinopileatus showed the most interesting skin whitening activity, followed by G. resinaceum, and by A. biennis (Table 1). For all strains, CC was the most appropriate carbon source for maximizing the potential production of bioactive small molecules with anti-aging activity. The use of OMWW as carbon source sligtly descreased the anti-tyrosinase potential of G. resinaceum and of A. biennis, while for P. citrinopileatus resulted in the complete loss of bioactivity (Table 1). The DPPH scavenging potential of the genarated extracts also drastically decreased with OMWW as carbon source, conluding that CC is the most appropriate carbon source for the production of anti-oxidant and anti-tyrosinase compounds from the aforementioned strains (Table 1).

Table 1. Anti-oxidant and anti-tyrosinase evaluation of fungal biomasses.

Carbon source*	Fungal extracts	% Tyrosinase Inhibition (± SD)		% DPPH Scavenging (± SD)	
		300 μg mL^{-1}	100 μg mL^{-1}	200 μg mL^{-1}	100 μg mL^{-1}
OOMW	**Abi**	23.22 ± 1.47	3.61 ± 3.16	0.46 ± 0.58	−1.19 ± 1.00
	Pci	3.42 ± 0.83	7.39 ± 3.73	0.57 ± 2.40	−1.34 ± 0.69
	Gre	22.13 ± 0.84	6.60 ± 2.39	0.58 ± 0.28	−0.23 ± 1.43
CC	**Abi**	25.20 ± 1.85	4.76 ± 2.56	24.01 ± 0.14	12.04 ± 0.25
	Pci	32.00 ± 0.92	27.13 ± 0.62	17.57 ± 0.41	10.40 ± 1.44
	Gre	27.25 ± 0.96	12.24 ± 0.20	16.71 ± 0.42	6.69 ± 0.62

*Cultivations were carried out in duplicates. **Abi**: *A. biennis*, **Pci**: *P. citrinopileatus*, **Gre**: *G. resinaceum.*

The enzymatic variability of the studied strains in regard to lignocellulose degradation was assessed by assembling 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 P. citrinopileatus and A. biennis, as shown in Table 2.

Table 2. Predicted protein sequences present in the *A. biennis* and *P. citrinopileatus* genome, relative to lignocellulose degradation.

Specific Activity		CaZy Modules	No of Predicted Sequences	
			A. biennis	*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 monooxygenases	AA 9, 10, 11, 13, 14	12	28

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. *P. citrinopileatus* was found to contain more copies of oxidative enzymes and endoxylanases than *A. biennis*. Especially for laccases, *Pleurotus* species are known to be potent producers [14], and *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 *G. resinaceum*, which is a strain with unknown genome sequence, were grown in different lignocellulosic materials as carbon sources, and the profile of secreted enzyme activities was explored in reaction supernatants.

All of the studied strains were found able to achieve complete breakdown of OOMW phenolic load. The maximum phenols degradation was observed by *P. citrinopileatus*, reaching up to 94.3 ± 0.9% together with 86.1 ± 0.6% color removal. *A. biennis* achieved up to 87.5 ± 1.5% phenols degradation and up to 71.5 ± 0.8% color removal, while for *G. resinaceum* phenols degradation reached 86.0 ± 2% and the respective color removal was 74.0 ± 0.1%. The time profile of laccase production is shown in Figure 1a. Surprisingly, *P. citrinopileatus* was found to be the least potent laccase producer, while *G. resinaceum* showed the highest laccase production, up to 2.1 ± 0.04 U mL^{-1}.

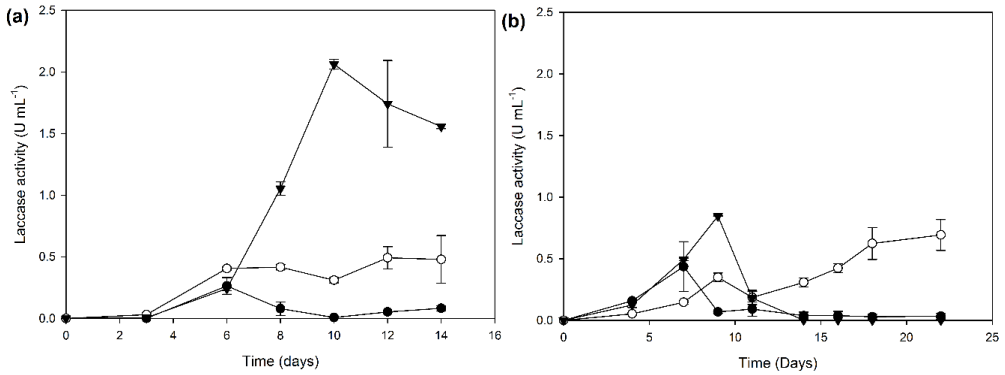

Figure 1. Laccase activity during the growth of P. citrinopileatus (black circles), A. biennis (white circles) and G. resinaceum (black inverted triangles), on OOMW (**a**) and CC (**b**).

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 culture. The results for laccase activity are shown in Figure 1b. Laccase activity for all strains was found to be significantly lower than when grown on OOMW, 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. Surprisingly, endocellulase and endoxylanase activities were not detected at any time point during the course of the fungal cultures. This results might be partly explained by the physiology of white-rot basidiomycetes, most of which are preferential lignin degraders [2].

Despite the absence of detectable endocellulase or endoxylanase activity, soluble reducing sugars were detected in the culture supernatants, up to 0.66 mg mL^{-1} ± 0.004 for *P. citrinopileatus*, 0.59 ± 0.04 mg mL^{-1} for *A. biennis* and 0.41 ± 0.01 mg mL^{-1} for *G. resinaceum*, indicating the presence of at least some enzyme activity on biomass polysaccharides. All the above data, together with the presence of several LPMO gene copies in the genomes of *P. citrinopileatus* and *A. biennis*, support the existence of potent LPMO activity. In order to detect LPMO activity, the three fungal strains were grown in microcrystalline cellulose (Avicel) as sole carbon source. After 15 days of growth, the supernatants were analyzed for LPMO activity. As shown in Figure 2, LPMO activity was detected in the supernatants of all fungal strains, albeit the presence of oxidized sugars [15] is more prominent in *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.

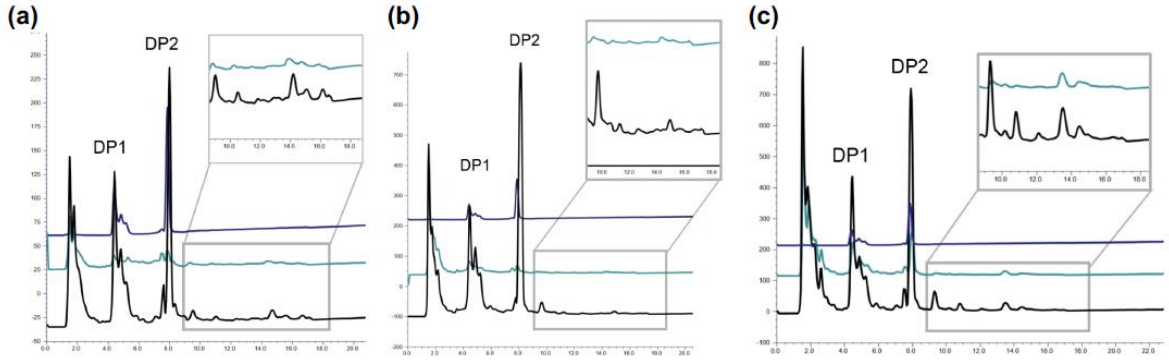

Figure 2. HPAEC-PAD chromatograms for the detection of LPMO activity. (**a**) *P. citrinopileatus*, (**b**) *G. resinaceum*, (**c**) *A. biennis*. *Dark blue line*: glucose and cellobiose controls, *light blue line*: reaction control with deactivated enzyme, *black line*: reaction of crude enzyme with PASC substrate.

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 agroindustrial byproducts: OOMW and CC. *P. citrinopileatus* showed the most interesting skin whitening activity while *A. biennis* the highest DPPH scavenging potential. For all strains, CC was the most appropriate carbon source for maximizing the production potential of bioactive small molecules. All strains showed significant degradative potential towards OOMW phenols, and by the production of laccase activity. The 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 the necessary genes for complete lignocellulose breakdown. Overall, all strains show promising antiaging properties and significant potential for the discovery of novel enzyme activities.

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