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Olive mill wastewater biodegradation potential of white-rot fungi – Mode of action of fungal culture extracts and effects of ligninolytic enzymes

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HIGHLIGHTS

- 49 white-rot strains (38 spp.) of basidiomycetes were evaluated for OMW degradation.
- No interaction between added catalase and fungal enzymes was observed in culture extracts.
- H_2O_2 addition resulted in drastic OMW's decolorization with no effect on phenolics.
- Heat-treatment of fungal extracts evidenced enzymatic oxidation of OMW's phenolics.
- Laccases added to OMW were reversibly inhibited by the effluent's high phenolic load.

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ABSTRACT

Forty-nine white-rot strains belonging to 38 species of Basidiomycota were evaluated for olive-mill wastewater (OMW) degradation. Almost all fungi caused high total phenolics (>60%) and color (⩽70%) reduction, while COD and phytotoxicity decreased to a lesser extent. Culture extracts from selected *Agrocybe cylindracea*, *Inonotus andersonii*, *Pleurotus ostreatus* and *Trametes versicolor* strains showed non-altered physicochemical and enzymatic activity profiles when applied to raw OMW in the presence or absence of commercial catalase, indicating no interaction of the latter with fungal enzymes and no competition for H_2O_2. Hydrogen peroxide's addition resulted in drastic OMW's decolorization, with no effect on phenolic content, suggesting that oxidation affects colored components, but not necessarily phenolics. When fungal extracts were heat-treated, no phenolics decrease was observed demonstrating thus their enzymatic rather than physicochemical oxidation. Laccases added to OMW were reversibly inhibited by the effluent's high phenolic load, while peroxidases were stable and active during the entire process.

1. Introduction

The operation of three-phase olive oil extraction systems results in the production of olive oil, solid pomace and an aqueous effluent, widely known as olive mill wastewater (OMW). OMW is a significant source of agro-industrial pollution due to its high organic load, polyphenols content, low molecular weight organic acids and salts (e.g. BOD: 4–9.5 g L^{-1}, COD: 5–18 g L^{-1}, LD_{50} toxicity for fish: 8.7%, polyphenols content: 1.5–12 g L^{-1}); moreover, the huge amounts of effluents generated annually (>15 M m^3 in the Mediterranean region only), and the seasonal operation and high territorial dispersal of olive oil mills further hinder their effective management (Aviani et al., 2012; Ntougias et al., 2006, 2013; Roig et al., 2006). In addition, extended land application of OMW could alter soil characteristics, invoke phytotoxicity phenomena and affect soil microbial communities (Karpouzas et al., 2010; Ouzounidou et al., 2012). On the other hand, treatment of OMW

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could lead to the development of various value-added products, e.g. natural antioxidants and enzymes, soil conditioners and fertilizers, substrates for growing microbial biomass, biopolymers and bioenergy (Aviani et al., 2010; Markou et al., 2012; Padovani et al., 2013; Romero-García et al., 2014; Zervakis et al., 1996, 2013).

Several physicochemical approaches have been employed for OMW treatment; however, their implementation is often met with various large-scale feasibility and cost-efficiency problems which could be further accompanied by other environmental or technical issues related to the formation of large amounts of toxic sludge, membrane fouling, emission of air pollutants, toxicity induced by radical species, etc.

Biological treatment of OMW by white-rot fungi constitutes an alternative approach for achieving their detoxification since they demonstrated the ability to degrade OMW's recalcitrant aromatic components, including monomeric and polymeric phenolic compounds, through the production of specific oxidative enzymes, i.e. laccases, lignin peroxidases and Mn-peroxidases (Cerrone et al., 2011; D'Annibale et al., 2004; Ntougias et al., 2012, 2013). Laccases, in particular, are copper-linked phenol oxidoreductases, which cause non-specific one-electron oxidation of aromatic compounds and reduce oxygen to water without requiring hydrogen peroxide; they can act on phenolic compounds, forming phenoxy radicals which can be either transformed to *p*-quinones or polymerized (Baldrian, 2006; de la Rubia et al., 2008). On the other hand, Mn-peroxidases require both hydrogen peroxide and manganese ions to transform phenolic compounds to radical species.

As previously mentioned, white-rot fungi have been experimentally used in OMW's treatment for several years; however, no massive screening of their efficacy to degrade OMW has been performed so far. Instead, such comparative evaluations were either restricted to a few species (Ntougias et al., 2012; Sayadi and Ellouz, 1993) or they were based on the assessment of one particular parameter (i.e. mycelium growth on solidified OMW-based media; Koutrotsios and Zervakis, 2014). In addition, little is known about the mechanisms by which extracellular ligninolytic enzymes from white-rot fungi interact with OMW's phenolics and hydrogen peroxide. Such information could substantially contribute to exploiting the biodegradation potential of highly effective fungal strains for detoxifying OMW.

In the present study, 49 wood-rot fungi were evaluated in terms of their ability to reduce OMW phenolics, color, phytotoxicity and organic load. Selected strains from this broad screening were used to further elucidate enzymatic mechanisms involved in the degradation of the effluent. Subsequently, heat treatment and addition of exogenous laccase, catalase and hydrogen peroxide on OMW-derived culture extracts were studied and interpreted in conjunction with phenol oxidases and peroxidases secreted in the OMW-based media.

2. Methods

2.1. Biological material studied

Forty-nine (49) wood-rot fungal strains assigned to 38 species and 26 genera of the phylum Basidiomycota were examined, i.e. *Abortiporus biennis* CCBAS 521, *Agrocybe cylindracea* LGAM 341, *Auricularia auricula-judae* LGAM 468, *Daedalea quercina* CCBAS 528, *Dichomitus squalens* CCBAS 750, *Flammulina velutipes* LGAM 804, *Fomitiporia punctata* CCBAS 662, *Ganoderma lucidum* CCBAS 707, *G. lucidum* LGAM 880, *Ganoderma pfeifferi* LGAM 892, *Ganoderma resinaceum* CCBAS-NA, *Ganoderma tsugae* LGAM 605, *Grifola frondosa* LGAM 807, *Hericium alpestre* CCBAS 654, *Hericium erinaceus* LGAM 311, *H. erinaceus* LGAM 312, *Hypholoma lateritium* LGAM 891, *Inonotus andersonii* CCBAS 557, *Inonotus nodulosus* CCBAS556, *Lentinellus castoreus* LGAM 898, *Lentinula edodes* LGAM 887, *L. edodes* LGAM 897, *Lentinus tigrinus* CCBAS 391, *Macrolepiota excoriata* LGAM 318, *Panus conchatus* LGAM 896, *Phanerochaete chrysosporium* CCBAS 571, *P. chrysosporium* LGAM 327, *P. chrysosporium* LGAM 990, *Pholiota nameko* LGAM 808, *Pleurotus djamor* LGAM 811, *Pleurotus eryngii* LGAM 219, *P. eryngii* LGAM 220, *Pleurotus levis* LGAM 218, *Pleurotus ostreatus* CCBAS 443, *P. ostreatus* LGAM 015, *P. ostreatus* LGAM 069, *P. ostreatus* LGAM 099, *P. ostreatus* LGAM 850, *Pleurotus pulmonarius* CCBAS 666, *Polyporus ciliatus* CCBAS 592, *Polyporus tuberaster* CCBAS 590, *Pycnoporus cinnabarinus* CCBAS 595, *P. cinnabarinus* CCBAS 787, *Stereum hirsutum* CCBAS 608, *Tapinella panuoides* LGAM 899, *Trametes hirsuta* CCBAS 610, *Trametes suaveolens* CCBAS 611, *Trametes versicolor* CCBAS 614 and *Tyromyces lacteus* CCBAS 616. All fungi are maintained in the culture collections of the Agricultural University of Athens, Laboratory of General and Agricultural Microbiology (LGAM), and of the Institute of Microbiology, ASCR (CCBAS).

2.2. Olive mill wastewater and growth of fungal strains

Olive mill wastewater (OMW) was obtained for two successive years from an olive oil mill using a three-phase centrifugation system (Kalamata, Greece). Fresh OMW was diluted to water 25% v/v, its pH was adjusted to 6.0 by the addition of CaO and, following centrifugation (4250*g*, 5 min), the supernatant was heat-sterilized (20 min, 121 °C, 1.1 atm). Fungal inocula (plugs of 6 mm diameter) were obtained from the peripheral part of 7-days-old mycelia growing on solidified OMW medium (25% v/v OMW supplemented with 1.5% w/v agar), and were then transferred into 250 ml Erlenmeyer flasks containing 50 ml OMW (25% v/v). Cultures were incubated at 25 °C, in the dark under static conditions; aeration was provided by agitating once per day. After 5 weeks, mycelium was harvested by filtration and its weight was measured after drying at 70 °C until constant weight was obtained.

2.3. Estimation of OMW's physicochemical parameters and phytotoxicity

Samples' pH was measured by a Scott Geräte TR156 pH-meter. Color was determined by measuring samples absorbance at 525 nm, whereas total phenolics were estimated by the Folin–Ciocalteu method against a syringic acid calibration curve and soluble COD (Chemical Oxygen Demand) was analyzed as previously described by Clesceri et al. (1998). Phytotoxicity was estimated by measuring the seed germination index (GI) of cress (*Lepidium sativum*): 25 cress seeds were placed onto a filter paper, moistened by the biotreated OMW (or by water for the control), incubated for 3 days in the dark, and then their germination percentage was calculated (Ntougias et al., 2012).

2.4. Determination of enzyme activities

Assessment of enzyme activities was carried out as previously reported (Ntougias et al., 2012). In brief, laccase activity (Lac) was measured colorimetrically at 425 nm by mixing 0.8 ml fungal extract with 0.4 ml 1.5 mM 2,2-amino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1.2 ml 0.1 M tartrate buffer at pH 4.5. The manganese-independent peroxidase (MnIP) activity was assessed at 590 nm by oxidizing 0.1 ml 1 mM 3-methyl-2-benzothiazoline hydrazone (MBTH) with 0.2 ml 25 mM 3-dimethylaminobenzoic acid (DMAB) in the presence of 0.66 ml fungal extract, 0.01 ml 10 mM H_2O_2 and 1 ml 0.1 M succinate-lactate buffer at pH 4.5. Calculations involved subtraction of background activity (estimated as above in the absence of H_2O_2). The manganese peroxidase (MnP) activity was estimated as reported for