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Efficient production of lignin-modifying enzymes and phenolics removal in submerged fermentation of olive mill by-products by white-rot basidiomycetes

Vladimir Elisashvili[a,b,*], Eva Kachlishvili[a,b], Mikheil D. Asatiani[a,b]

[a] Agricultural University of Georgia, Tbilisi, Georgia
[b] Center for Microbial Biotechnology, Tbilisi, Georgia

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ABSTRACT

The ability of seven distinct species of white-rot basidiomycetes (WRB) to produce lignin-modifying enzymes (LME) and reduce high phenolic content was assessed during submerged fermentation of oil mill by-products. *Cerrena unicolor* strains appeared to be the outstanding producers of laccase (56.3–78.5 U mL^{-1}) and MnP (1.03–2.76 U mL^{-1}) in the fermentation of olive pomace (OP), and laccase (26.9–93.8 U mL) in the fermentation of olive tree sawdust. Diluted 50% olive mill wastewater (OMW) can be used as a nutrient medium for laccase and MnP production but its supplementation with additional carbon and nitrogen sources and Cu^{2+} and $Mn^{2=}$ promoted the 3-, 28- and 18-fold increase of *C. unicolor* biomass and volumetric laccase and MnP activities, respectively, as well as 3- and 17-fold increase of *Pleurotus ostreatus* biomass and laccase activity, respectively. The tested fungi successfully reduced the phenolics content in the submerged fermentation of OP (72.0–81.3%) and OMW (62.4–83.5%). The laccase role in the OP phenolics reduction was proved through the treatment of 10% (wet weight) OP with commercial and lab-generated laccases preparations and reduction of total phenols by 66–70% during 24 h incubation. This work indicated that the olive mill by-products fermentation by individual substrate-specific WRB is an economically feasible and promising alternative for the simultaneous production of LME, cellulase, xylanase and detoxified materials suitable for further exploitation in biotechnological and agronomic applications.

1. Introduction

Agro-industrial residues produced in huge amounts and generally accumulated in the environment could be used as raw materials for the production of a wide range of value-added products. Nowadays, production of lignin-modifying enzymes (LME) through the submerged and solid-state fermentation of lignocellulosic materials by white-rot basidiomycetes (WRB) is under considerable attention. These fungi secrete several extracellular enzymes that are essential for lignin degradation: lignin peroxidase (EC 1.11.1.14), manganese-dependent peroxidase (MnP) (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16) and laccase (EC 1.10.3.2). These enzymes are highly hopeful for their applications in the chemical, fuel, food, agricultural, paper, textile and cosmetic industries as well as in bioremediation of environments polluted by persistent and toxic compounds (Yadav and Yadav, 2015). Therefore, the demand for the development of efficient and low-cost technologies of LME production is ever increasing. To solve this issue, one of the appropriate approaches is to utilize the potential of food industry wastes/by-products, some of which contain significant concentrations of required nutrients and inducers ensuring an abundant growth of WRB and efficient production of LME (Kachlishvili et al., 2014; Elisashvili et al., 2017; Zhao et al., 2017).

Olive oil manufacture from olives results in the generation of huge amounts of by-products, in the three-phase systems the olive pomace (OP) and the olive mill wastewater (OMW) and in the two-phase systems the two-phase olive mill waste (Dermeche et al., 2013). In addition, the olive-tree trimmings represent a great volume of biomass in Mediterranean countries. These by-products create significant environmental problems related to their disposal. In particular, OMW is a highly polluting effluent containing chemical oxygen demand 30–320 g/l, biological oxygen demand 32–132 g l^{-1} and total phenols 0.63–5.45% (Dermeche et al., 2013). Due to high organic load and content of monomeric and polymeric phenolic and aromatic compounds, the OMW causes a phytotoxic effect and inhibition of microbial

* Corresponding author. Agricultural University of Georgia, Tbilisi, Georgia.
E-mail address: v.elisashvili@agruni.edu.ge (V. Elisashvili).

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growth (Casa et al., 2003; Ntougias et al., 2012; Daâssi et al., 2016). Therefore, reduction of the toxic substances contained in oil mill by-products is a major issue and many research efforts have been undertaken to develop cost-effective and environmentally-safe disposal methods. For this reason, various physicochemical and biological approaches have been employed to valorize them and to recover valuable products such as dietary fibers, animal feed, phenolic compounds, biofuel, biogas, enzymes, polymers and other (Crognale et al., 2006; Dermeche et al., 2013). Water extracts of the dry olive-mill residue appeared to be a suitable growth medium for laccase and MnP production by several WRB without supplementation of nutrients or inducers (Sampedro et al., 2012; D'Annibale et al., 2014). Solid waste of olive oil extraction process was used as a growth medium for mushroom production (Zervakis et al., 1996) while the partly diluted OMW was used as a wetting agent during the oyster mushroom and shiitake cultivation (Kalmıs et al., 2008). The treatment of OMW by WRB decreased phenolics content and at a suitable dilution, it was utilized as an organic amendment in agriculture (Casa et al., 2003; Daâssi et al., 2016).

Despite the progress in oil mill by-products treatment, the search for novel strains of fungi able to withstanding and eliminate the toxic effects of phenolic compounds are of significant scientific and industrial interest. Few studies were performed in submerged and solid-state cultivation of WRB in order to promote the LME production on the OMW-based media (Fenice et al., 2003; Jaouani et al., 2003, 2005; Chakroun et al., 2009; Goudopoulou et al., 2010; Mann et al., 2015; Zerva et al., 2017). However, information on fermentation of the OP or olive cake to produce LME by WRB is limited (Sampedro et al., 2007, 2012; Ruiz-Rodríguez et al., 2011; Neifar et al., 2013; Reina et al., 2013) and to the best of our knowledge, no report is available on LME production in fermentation of olive tree wood sawdust. The aim of the present study was to exploit the biodegradation abilities of seven WRB species with earlier proved high activity of LME (Elisashvili and Kachlishvili, 2009; Kachlishvili et al., 2014; Elisashvili et al., 2017) to reduce high phenolic content in OP and OMW and to offer an economically feasible alternative for the simultaneous production of LME and detoxified OMW which can be further used in biotechnological and other processes. The ability of WRB to simultaneously produce cellulase and xylanase, the effect of several supplements on enzyme production in OMW-based medium, and the potential of isolated laccases to act as biocatalysts for phenolics reduction were also assessed.

2. Materials and methods

2.1. Materials, organisms and inocula preparation

Three olive pomaces (OP) samples and olive mill wastewater (OMW) were provided by the company “Escobedo y García Asesores s.l”, Jaén (Spain). Well mixed small portions of OP samples I, II and III preliminarily were dried in an oven at 60 °C to the constant weight to establish their dry biomass content - 29.2, 40.3 and 16.7%, respectively. However, in the fermentation experiments, initial wet samples were used on the base of their dry matter. Dried olive tree wood sawdust (particle size < 1 mm) was obtained from the same company. Mandarin peels from the local market were oven-dried at 50 °C and ground to powder in a laboratory mill prior to addition to the nutrient medium.

The following fungal strains deposited in the basidiomycetes culture collection of the Agricultural University of Georgia were used in this study: *Cerrena unicolor* BCC 301, *C. unicolor* BCC 302, *C. unicolor* BCC 303, *Fomes fomentarius* BCC 38, *Ganoderma lucidum* BCC 447, *Pleurotus ostreatus* BCC 2175, *Pycnoporus coccineus* BCC 310, *Trametes trogii* BCC 146, and *T. versicolor* BCC 13. The fungal inocula were prepared by growing their mycelium on a rotary shaker in 250 mL flasks containing 100 mL of the following medium (per L): 15 g glucose, 3 g peptone, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 3 g yeast extract. Cultures were incubated at 27 °C and 150 rpm for 7 days. The grown fungal biomasses were homogenized in a Waring laboratory blender and used as an inoculum for submerged cultures.

2.2. Cultivation conditions

The submerged cultivation of fungi was performed in the rotary Innova 44 shakers (New Brunswick Scientific, USA) at 27 °C and 160 rpm. The homogenized mycelium (5 mL) was used to inoculate the 250-mL flasks containing 50 mL of the basal medium (per L): 1.0 g KH_2PO_4, 0.5 g $MgSO_4$ x $7H_2O$, 3.0 g yeast extract, 0.3 mM $CuSO_4$ x $5H_2O$, 0.1 mM $MnSO_4$, pH 6.0. To test a suitability of OP for fungi cultivation and LME production and to elucidate the limits of their tolerance to this material, the basal medium was supplemented with 1, 2 and 4% dry weight of OP and 0.3% peptone as an additional nitrogen source. To evaluate a suitability of OMW for the selected fungi cultivation and LME production, 50% OMW (v/v, diluted with the distilled water) without and with several supplements was used as a growth medium. In the final set of experiments, a capability of WRB to produce LME was compared in media containing mandarin peels, olive tree sawdust, OP, and OMW. In this case, the basal medium was supplemented with an additional nitrogen source, peptone at the concentration of 3 g L^{-1}.

The media pH was adjusted to 6.0 prior to sterilization and all submerged cultivations were carried for 14 days. At predetermined time intervals (usually after 6, 10, and 14 days), 1 mL of culture was sampled and solids were separated by centrifugation (Eppendorf 5417R, Hamburg, Germany) at 10,000 g for 5 min at 4 °C. The supernatants were analyzed for pH, enzyme activities, and phenols.

All experiments were performed twice using three replicates at each time point. All results were expressed as the mean ± SD (standard deviation) with only $p \leq 0.05$ considered as statistically significant. The analyses were carried out using Microsoft Office Excel software.

2.3. Enzymatic treatment of the OP

Laccase preparations isolated from the culture liquids of *C. unicolor* 303 and *T. trogii* 146 by precipitation with ammonium sulfate at 75% saturation were employed in this study to perform the OP treatment. For comparison, the commercial granulated laccase from Genencor (USA) was also used. The treatment with enzymes was performed using the following conditions: 10 g of the wet sample III in the 100 mL flasks were diluted with 10 mL 0.05 M acetate buffer, the reaction mixture pH was adjusted to 5.0 and incubated in the shaker 1 h at 27 °C and 160 rpm. Then the laccase preparations were added to the reaction mixtures to the final concentration of 1 U mL^{-1} and the incubation continued for 24 h. Controls containing the heat-denatured enzymes were performed under the same incubation conditions. After incubation, the flasks content was centrifuged at 8000 g for 15 min and the supernatants were used for the measurement of phenolics content.

2.4. Analytical methods

The grown fungal biomass was measured gravimetrically after fungi cultivation in the OMW-based medium by recovering mycelium with centrifugation of whole cultures at 8000 g for 20 min and drying at 70 °C for 24 h. The total phenolic content in the supernatants was analyzed by the Folin–Ciocalteu method (Fluka, Switzerland). According to this method, 500 μL Folin–Ciocalteau (4-fold-diluted) phenol reagent was added to 100 μL of properly diluted samples. After 5 min, 500 μL 10% sodium carbonate was added and after incubation at room temperature for 30 min the absorbance was measured at 725 nm against a blank. Phenolics concentration was expressed in ppm of gallic acid equivalents, using the appropriate calibration curve.

Laccase activity was determined spectrophotometrically (Camspec M501, Cambridge, UK) at 420 nm as the rate of 0.25 mM ABTS (2,2′-azino-bis-(3-ethylthiazoline-6-sulfonate)) oxidation in 50 mM Na-