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Screening of eucalyptus wood endophytes for laccase activity

Úrsula Fillat[a,*], Raquel Martín-Sampedro[a], David Macaya-Sanz[b], Juan A. Martín[b], David Ibarra[a], María J. Martínez[c], María E. Eugenio[a]

[a] INIA-CIFOR, Forestry Products Department, Cellulose and Paper Laboratories, 28040 Madrid, Spain
[b] Department of Natural Systems and Resources, School of Forest Engineers, Technical University of Madrid, 28040 Madrid, Spain
[c] Centro de Investigaciones Biológicas (CIB), CSIC, Environmental Biology Department, 28040 Madrid, Spain

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ABSTRACT

New lignin-degrading microorganisms and their enzymes can contribute to more efficient and environmentally sound use of lignocellulosic biomass in future biorefineries. In view of this, 127 endophytic fungi were isolated from eucalyptus trees. Strains were tested for their ability to produce ligninolytic enzymes in agar-plate medium containing ABTS, and 21 showed positive ABTS-oxidation. Positive strains in liquid medium confirmed that five produced laccase but no peroxidase activity. These strains were identified as *Hormonema* sp. and *Pringsheimia smilacis*, of the family Dothioraceae, *Ulocladium* sp. (Pleosporaceae) and *Neofusicoccum luteum* and *Neofusicoccum australe* (Botryosphaeriaceae). Laccase production by fungi of the family Dothioraceae is described here for the first time.

To increase laccase production from these fungi, copper sulphate and ethanol were assayed as inducers. An increase in laccase activity of 85% was found for *Hormonema* sp. and *N. luteum* and 95% for *N. australe*. No increase was obtained for *P. smilacis*. A maximum temperature of 40 °C is recommended for possible biotechnological applications with these four strains at pH 2 for *N. luteum* and *N. australe*, pH 4 for *Hormonema* sp. and pH 4–5 for *P. smilacis*.

1. Introduction

Wood is the most abundant biopolymer in nature and consists of three main polymeric components: cellulose, hemicelluloses, and lignin [1]. Lignin gives plants mechanical resistance and, due to its chemical complexity, very few organisms are capable of degrading it [2]. Removal of lignin is a key factor in the pulp and paper industry and the biorefineries of the future for producing value-added chemicals, materials and biofuels based on cellulose and hemicelluloses. In the development of biorefineries, biotechnology could play an important role by providing efficient and ecofriendly biocatalysts for lignin degradation [3].

Bacteria and fungi, notably wood-decaying fungi and, in particular, white-rot fungi, are among a number of microorganisms that are capable of efficiently depolymerising and mineralising lignin [2]. These fungi have developed a complex system of lignocellulose-degrading enzymes comprising peroxidases, oxidases and reductases and low molecular mass compounds that mediate the action of these enzymes [2]. This ability makes white-rot fungi useful for a wide range of biotechnological applications in industrial uses of lignocellulosic biomass [2]. The main oxidoreductases studied in biotechnological applications are laccases, which act on phenol and aromatic compounds through the reduction of molecular oxygen to water [3]. Basidiomycota is recognised as the most significant phylum of white-rot fungi that secretes these enzymes [4]. However, some species in Ascomycota, such as *Myceliophthora thermophila*, also produce laccases of major interest for industry because of their high thermal stability and activity at alkaline pH [5].

Most studies of wood-decaying fungi are based on advanced stages of wood degradation. However, some endophytic fungi could be involved in triggering the development of early stages of wood decay. In nature, endophytes inhabit asymptomatic plant tissues, living in symbiosis with their hosts. These fungi represent an enormous fungal diversity of as yet unknown magnitude and ecological functions [6,7]. The discovery of their taxonomy and functions is a great challenge that would fall within the scope of recent initiatives which propose the study of the diversity and effects of microbial ecosystems in a unified, coordinated and interdisci-

* Corresponding author.
E-mail addresses: fillat.ursula@inia.es, yurs_79@yahoo.es (Ú. Fillat), martin.raquel@inia.es (R. Martín-Sampedro), david.macaya.sanz@gmail.com (D. Macaya-Sanz), juan.martin.garcia@upm.es (J.A. Martín), ibarra.david@inia.es (D. Ibarra), mjmartinez@cib.csic.es (M.J. Martínez), mariaeugenia@inia.es (M.E. Eugenio).

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plinary way [8,9]. After the death of the plant or any of its organs, some endophytes seem to move from a dormant state to become key primary colonisers involved in the degradation of plant tissues [10]. In this phase, these fungi would have an advantage over other competing saprophytes, having gained the niche before decomposition begins [11]. As a result of co-evolutionary processes, certain endophytes have developed complex enzymatic systems and metabolites [12,13], becoming highly specialised in degrading the wood polymers of their particular host species, especially during the initial stages of wood decomposition. Interestingly, other specialised microorganisms, including dark septate endophytes and arbuscular mycorrhizal fungi, modulate lignin biosynthesis in living plants (mainly in above-ground tissues) through their interaction with plant roots, and this way they can alter plant resistance to pest and pathogens [14]. Other studies have emphasized the conditioning effect of environment on the outcome of plant-endophyte interactions, and this way the non-static nature of these interactions [15,16]. However, little is known about the temporal and spatial variations in endophyte communities in large, long-lived forest trees.

It is necessary to further explore the role of wood-inhabiting fungi in wood biodegradation and study their ligninolytic enzymes, as they could be an important alternative for degrading lignin or other recalcitrant compounds that cause environmental problems. Technological application of these fungi in industrial processes, either before or in combination with secondary saprophytes, could improve current technological performance.

The aim of this study was to evaluate the production of ligninolytic enzymes in liquid medium secreted by endophytic fungi isolated from eucalyptus wood as an alternative to the more studied wood-rotting fungal species. To reach this goal, after a preliminary screening on solid medium containing ABTS, strains showing ABTS oxidation were identified and production of ligninolytic enzymes, laccases and peroxidases in liquid medium was analyzed. To increase laccase production from the selected fungi, the medium was supplemented with copper sulphate and ethanol as inducers. The characterization (stability and optimum pH and temperature) of the laccases secreted by these fungi were also studied for different biotechnological applications.

2. Materials and methods

2.1. Fungal isolation

A total of 127 strains of endophytic fungi were isolated from *Eucalyptus globulus* trees in five regions of Spain: Cantabria (coded CA at the beginning of the strain name), Asturias (AS), Seville, (SE), Extremadura (EX) and Toledo (TO).

For endophyte isolation, four twigs approximately 2 cm in diameter were collected from 1–3 trees from each sampling location. After surface sterilisation with 70% ethanol and 4% bleach and removal of the outermost bark, five explants per twig were cultured in the following media: Malt Extract Agar (MEA), Potato Dextrose Agar, Rose Bengal Chloramphenicol Agar, Yeast Extract Agar, and Sapwood Agar (coded M, P, R, Y, and S in the penultimate position of the strain name). The first four media were prepared following the manufacturer's instructions (Cultimed, Panreac, Barcelona, Spain). Sapwood agar was prepared by mixing 50 g ground, dried and autoclaved eucalyptus twigs with 7.5 g agar in 500 mL water. Explants were axial twig slices approximately 2 mm thick, including phloem and xylem tissue. After two weeks of incubation in a dark chamber at 25 °C, growing fungal strains were transferred to separate plates containing MEA.

2.2. Primary ligninolytic activity screening in solid medium

Extracellular enzymatic activities were firstly assayed at 22 °C for three days in Petri dishes with a basal solid medium (KH_2PO_4, 1 g/L; $C_4H_{12}N_2O_6$, 0.5 g/L; $CuSO_4{\cdot}5H_2O$, 0.001 g/L; $MgSO_4{\cdot}7H_2O$, 0.5 g/L; $Fe_2(SO_4)_3$, 0.001 g/L; $CaCl_2{\cdot}2H_2O$, 0.01 g/L; $MnSO_4{\cdot}H_2O$, 0.001 g/L; and yeast extract, 0.01 g/L) containing glucose, 4 g/L; agar, 16 g/L; and 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, from Roche Applied Sciences) [17]. ABTS is a very sensitive substrate that allows rapid screening of fungal strains producing extracellular ABTS-oxidising enzymes (such as laccases and peroxidases) by means of color reaction (dark green color in solid media) [18]. *Pycnoporus sanguineus* and *Trametes* sp. I–62, both basidiomycete white-rot fungi obtained from the IJM collection (Instituto Jaime Ferrán de Microbiología—CIB, CSIC), were used as positive controls for wood-rot fungi. Plates were inoculated with agar disks of active mycelia previously cultured in malt extract agar.

2.3. Identification of fungal strains

Strains showing laccase activity in solid medium were identified by their specific Internal Transcribed Spacer regions (ITS1 and ITS2) of ribosomal DNA isolated following the method described by Cenis [19], with minor modifications. A Polymerase Chain Reaction (PCR) was then performed to amplify ITS regions. The thermal protocol for amplification was 4 min at 94 °C, 30 cycles of 30 s at 94 °C, 45 s at 55 °C, and 1 min at 72 °C, followed by a final extension of 5 min at 72 °C. In a final volume of 25 μL, the PCR mix included 5–10 ng DNA template, 0.625 units of Taq (Bioline, London, UK), and a final concentration of 0.2 μM of each primer [ITS1-F [8] and ITS4 [9]], 0.2 mM each dNTP, and 1 mM $MgCl_2$.

After checking for positive amplification in agarose gel (2%) with SYBR® Safe staining (Life Technologies, Carlsbad, CA, USA), PCR product was purified by enzymatic incubation (20 min at 37 °C followed by 15 min at 85 °C for enzyme deactivation). Enzymes used were Exonuclease I (Exo I; Thermo Scientific, Waltham, MA, USA) and Calf Intestine Phosphatase alkaline (CIP; AppliChem, Darmstadt, Germany). Final concentrations were 1.4 units/μL for Exo I and 0.14 units/μL for CIP. PCR purified products were delivered to external facilities (Secugen, Madrid, Spain) for Sanger sequencing in both directions.

For each strain, two sequences were obtained. These were aligned to achieve one consensus sequence for each strain. The BLAST (MEGAblast algorithm) online programme, by GenBank (NCBI, USA), was used to find the most similar sequence (highest bit score) within this database.

2.4. Secondary ligninolytic activity screening in liquid medium

Only strains showing positive ABTS oxidation in solid medium were cultivated in liquid medium for 21

days at 22 °C for ligninolytic enzyme determination. Pre-inocula were prepared by homogenising the mycelium from three-day-old fungal cultures in MEA into modified Kirk medium (sacarose, 10 g/L; KH_2PO_4, 2 g/L; aspargine, 1 g/L; tiamine·HCl, 1 mg/L; $MgSO_4{\cdot}7H_2O$, 0.5 g/L; veratryl alcohol, 57 μL/L; $CaCl_2{\cdot}2H_2O$, 0.1 g/L; CuSO4, 0.27 mM; wheat straw, 2 g/L; and trace elements: nitriloacetic acid, 1.5 g/L; $MnSO_4{\cdot}H_2O$, 0.5 g/L; NaCl, 1 g/L; $FeSO_4{\cdot}7H_2O$, 0.1 g/L; $CoCl_2$, 0.1 g/L; $ZnSO_4{\cdot}7H_2O$, 0.1 g/L; $CuSO_4{\cdot}5H_2O$, 0.01 g/L; $AlKSO_4{\cdot}12H_2O$, 0.01 g/L; H_3BO_3, 0.01 g/L; $NaMoO_4{\cdot}2H_2O$, 0.01 g/L). After three days, 4 mL pre-inoculum was inoculated in 500 mL Erlenmeyer flasks containing 200 mL of this medium. Culture supernatants were periodically taken from two replicate flasks to measure enzymatic activities. Mycelium was separated from the fungal culture by