Appl Biochem Biotechnol (2011) 163:415–422
DOI 10.1007/s12010-010-9049-2

Enhanced Laccase Production in White-Rot Fungus *Rigidoporus lignosus* by the Addition of Selected Phenolic and Aromatic Compounds

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Received: 12 April 2010 / Accepted: 19 July 2010 /
Published online: 15 August 2010

Abstract The white rot fungus *Rigidoporus lignosus* produces substantial amounts of extracellular laccase, a multicopper blue oxidase which is capable of oxidizing a wide range of organic substrates. Laccase production can be greatly enhanced in liquid cultures supplemented with various aromatic and phenolic compounds. The maximum enzyme activity was reached at the 21st or 24th day of fungal cultivation after the addition of inducers. The zymograms of extracellular fluid of culture preparation in the presence of inducers, at maximum activity day, revealed two bands with enzymatic activity, called Lac1 and Lac2, having different intensities. Lac2 band shows the higher intensity which changed with the different inducers. Laccase induction can be also obtained by adding to the culture medium olive mill wastewaters, which shows a high content of phenolic compounds

Keywords *Rigidoporus lignosus* · Laccase · Induction · Aromatic compounds · Wastewater

Introduction

Laccases (benzendiol:oxygen oxidoreductase E.C.1.10.3.2) are glycosilated polyphenoloxidases widely distributed in plants, fungi, insects and bacteria [1, 2]. Laccases are a family of multicopper oxidase that catalyze the one-electron oxidation of a wide range of aromatic substrates, including mono-, di- and polyphenols, substituted phenols, aromatic amines and lignines with the concomitant four-electron reduction of molecular oxygen to water [3, 4]. However, it has been demonstrated that, in the presence of oxidizable low molecular weight compounds, the so-called mediators, laccases, are able to oxidize a wide range of other aromatic compounds, such as polycyclic aromatic hydrocarbons [5, 6]. Several of these

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enzymes have been purified and characterized biochemically [7, 8] and a lot of the genes encoding the protein have been cloned and expressed in heterologous organisms [9, 10]. Recently, we have reported the crystallization and X-ray structure determination of fully copper-complemented laccase from white-rot basidiomycetes *Rigidoporus lignosus* (Rl) [11]. Because of the remarkable catalytic properties of laccases toward a broad range of substrates, these enzymes offer several advantages of great interest for industrial and biotechnological applications [12–14]. Therefore, there are attempts to use laccases for delignification of woody fibres [15], decolourisation of synthetic dyes [16, 17], detoxification of organic pollutants [18], including olive mill wastewater (OMW) [19, 20], and development of laccase-based amperometric biosensors for the determination of phenolic compounds [21, 22]. Considering these possible applications of laccases in biotechnology, any attempt to improve their production from fungal source would be worth investigating. Because these investigations usually require a large amount of enzyme, its yield can be very simply increased by the addition of inducers into the culture medium [23, 24]. In a previous paper, we described an implemented process for the production of laccase from Rl, by which this extracellular enzyme can be synthesized in an inexpensive medium. Laccase and its isoforms, called Lac1 and Lac2, were purified to apparent electrophoretic homogeneity and some of their structural and kinetic parameters were determined [25].

In the present study, several putative laccase inducers, such as selected synthetic phenolic compounds and natural product from OMW, having a high concentration of phenols, were tested for their ability to stimulate laccase production in the Rl cultures.

Materials and Methods

Materials

Phenolic standards were purchased from Sigma. OMWs used were obtained from a three-phase decanter manufacturer located in Sicily, Italy. Samples were collected, transported to the laboratory and freeze-dried to guarantee the chemical stability of this product over time [26]. Before the addition to the culture flask, the OMW samples were defrosted and then centrifuged to eliminate the insoluble residue, and the clarified solution was diluted to 10% with water and sterilized by filtration.

Culture Conditions

Culture medium, concentration of inducers, incubation time and temperature were determined before the experiments. Mycelial plugs taken from a preculture sample of native Rl K1 strain [27] were added to 500-ml Erlenmayer flasks containing the basic synthetic liquid medium (25 mM nitrogen provided by ammonium tartrate and 0.1% glucose as carbon source). The medium, buffered at pH 7 with 10 mM Tris–HCl, was supplemented with $CuSO_4$ (500 μg/l). Cultures were incubated at 30 °C, under static conditions, for 24 days. The compounds tested as potential inducers were dissolved in the incubation medium, sterilized by filtration and added to actively growing fungal cultures on the fourth day of cultivation at 1-mM concentration. The choice of inducers has been carried out on the basis of their chemical structure and redox potential. Likewise, clarified OMW solution, diluted to 10% with water, was sterilized by filtration and added to Rl culture at the fourth day of cultivation. Culture medium, incubation time and temperature were the same as described above.

Phenolic Compounds Determination

The concentration of total phenolic compounds was determined in the OMW solution using the Folin–Ciocalteu reagent and gallic acid as standard [28].

Enzyme Assay

Samples of laccase from Rl extracellular fluid (E.F.) obtained after the centrifugation of culture medium were harvested and their activity was assayed spectrophotometrically at 30 °C following the absorbance increase of syringaldazine at λ=526 nm (ε_{526}= 65.000 M^{-1} cm^{-1}). The reaction mixture contained 0.03 mM syringaldazine (dissolved in DMSO) as substrate, 10 mM Tris–HCl buffer, pH 7.0, 10 μl of E.F., in the final volume of 1.5 ml. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of substrate per minute at 30 °C. The activities were expressed as units ml^{-1}.

Protein Determination

Protein concentration of Rl E.F. was determined by bicinchoninic acid assay which did not interfere with the presence of phenolic compounds [29]. The bovine serum albumin was used as standard. The results were reported as total proteins per microgram per litre of E.F.

Native PAGE

Native PAGE analysis of the proteins was performed by the method of Laemmli [30] in 12% polyacrylamide gels on aliquots of E.F. at the maximum activity day. Protein bands with laccase activity were visualized by soaking the gels in 50 mM sodium acetate buffer (pH 4.5) containing 2 mM guaiacol as substrate.

Results and Discussion

Laccase Induction by Aromatic Compounds

The constitutive capability of Rl K1 strain to produce extracellular laccase under a submerged condition can be greatly enhanced by the addition of selected aromatic and phenolic compounds to basic growth medium. Nevertheless, there is a wide-ranging variation in the optimal time-point at which inducing compounds should be added to nutrient medium to effectively produce laccase. Highest enzyme activity was observed in *Botryosphaeria rhodina* when veratryl alcohol was added to the nutrient medium at the beginning of fermentation [31], whereas in *Trametes modesta* the optimal time-point was reached 5 days after the addition of various inducers [32]. Figure 1 shows the *R. lignosus* laccase (RlL) activity at their maximum level, which occurred at 21–24 days after the addition of inducers. The highest RlL activity, with respect to the control, was observed in the cultures supplemented with phenylhydrazine, (120.0 U ml^{-1}), OMW (82.0 U ml^{-1}), guaiacol (77.5 U ml^{-1}) and 4-methylcatecol (75.0 U ml^{-1}). Lower effects were obtained with other compounds such as catecol > pyrogallol > hydroquinone > ferulic acid > catechin > caffeic acid > vanillic acid > gallic acid. No activity was obtained by adding chloro- and nitro-substituted phenols (not shown).

It is worth noting that most inducers of RIL are also substrate of the enzyme to be produced. In particular, the phenolic compounds that stimulated laccase production in Rl

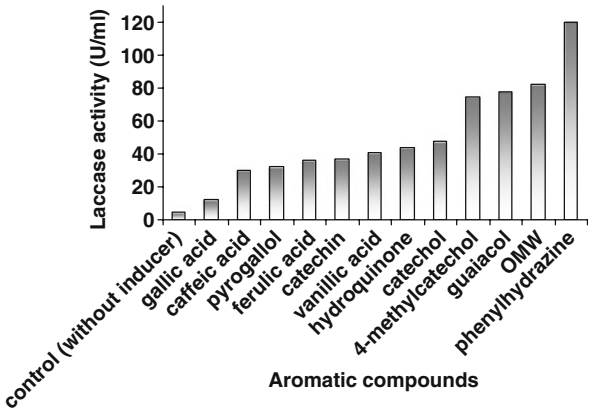

Fig. 1 Effect of aromatic compounds on maximum laccase activity. *Values* represent the mean of triplicate samples. Standard deviations were less than 5%

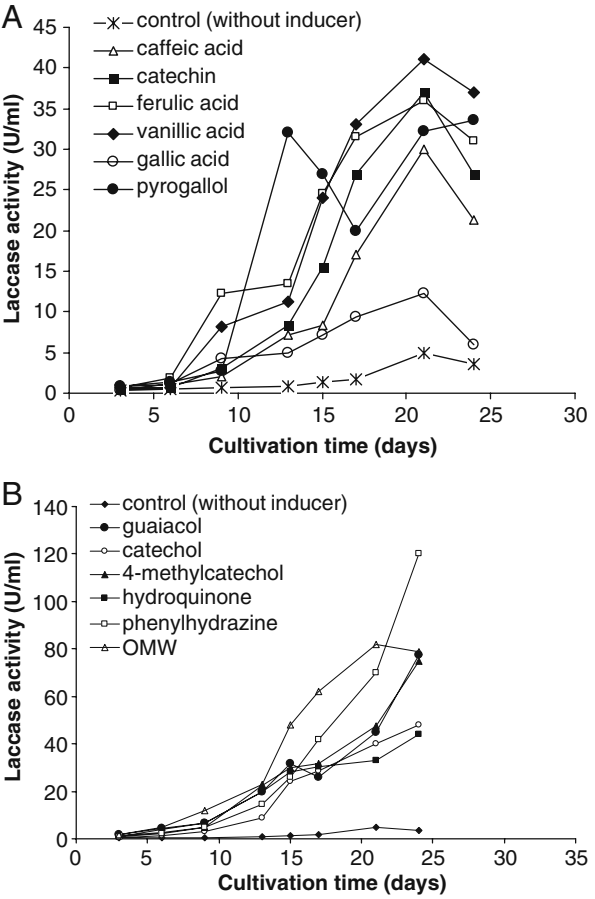

Fig. 2 **a**, **b** Time course of laccase activity in extracellular fluid of *R. lignosus* culture after addition of various inducers (1 mM). Maximum enzyme activity at 21st day (**a**) and at 24th day (**b**), respectively. *Values* are the mean of triplicate samples. Standard deviations were less than 5%

are those substituted with electron-donating groups such as methoxy, methyl, amino and hydroxyl, having a redox potential ($E°$) lower than 500 mV [33]. Conversely, the compounds with electrowithdrawing radicals, such as 4-chlorophenol and 4-nitrophenol having the relatively high $E°$ (653 and 924 mV, respectively) [34], did not stimulate induction of the RlL which has an $E°$ of 730 mV. Consistently, our results support the notion that the efficiency of the inducers to enhance the laccase production strongly depends on their redox potential difference with the enzyme.

Time Course of Enzyme Production and Protein Secretion

The time course of RlL extracellular production, after the addition to the culture medium of selected aromatic compounds at a final concentration of 1 mM, indicated that during growth the enzyme activity appeared after 48 h of incubation. Afterwards, it increased

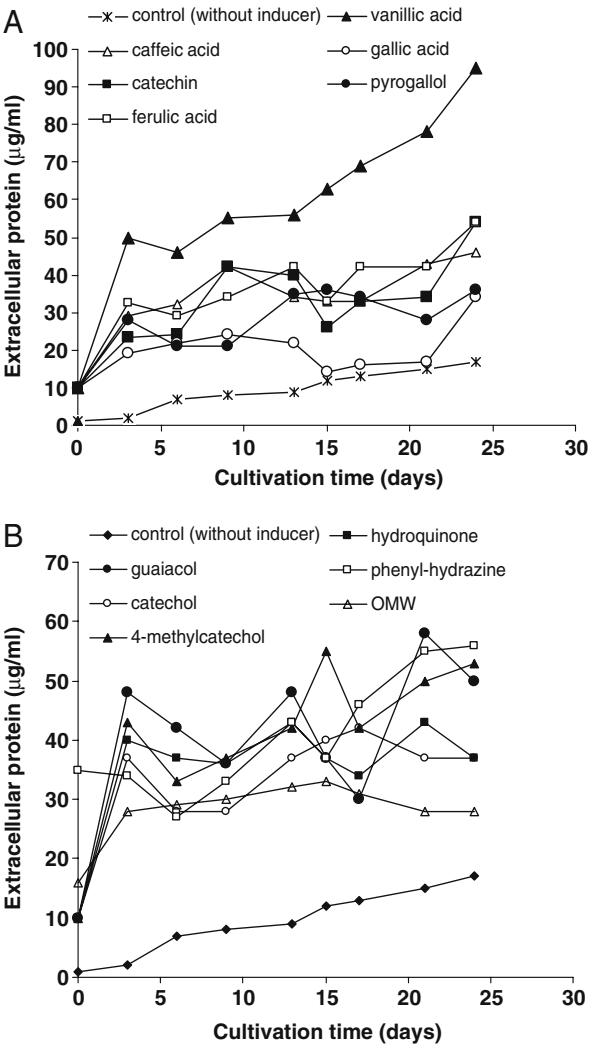

Fig. 3 **a**, **b** Time course of total proteins secretion in the extracellular fluid of *R. lignosus* culture in response to inducers. *Values* are the mean of triplicate samples. Standard deviations were less than 5%

progressively and reached a peak level after 21–24 days, respectively (Fig. 2a, b), in response to the specific inducer.

The behaviour of the RIL production after the addition of different aromatic compounds suggests a different activation of laccase gene which has been demonstrated to occur in many fungal species at the transcriptional level [35, 36]. For some inducers, a moderate decrease of RIL production towards the end of the experiment was observed (Fig. 2a). This decrease may be due either to the release of proteases during autolysis of the fungal pellets, which can degrade extracellular proteins, or to the inhibitory effect of the laccase by-products at the late-growth phases. This late finding is in agreement with other results which have demonstrated that the products of laccase reaction inhibit the enzyme itself. In fact, laccase reaction products spontaneously polymerized to form water-insoluble aggregates which can inhibit the enzyme by absorption or binding. We also determined the time course of protein secretion in the extracellular fluid of the Rl culture, supplemented with various inducers. As reported in Fig. 3a, b, protein concentration in the growth medium showed a progressive increase toward the end of cultivation, reaching the maximum after 21–24 days.

Laccases Isoenzymes Synthesis

In order to identify the inducible forms of RIL, the native PAGE analysis of protein in E.F. of Rl culture in the presence of inducers at maximum activity day was performed. The zymograms of this preparation were carried out by activity staining with guaiacol. Only two bands with enzymatic activity were revealed as having different intensities, namely, laccase 1 (Lac1, minor band) and laccase 2 (Lac2, major band), the latter with higher electrophoretic mobility. These two bands correspond to two isoforms of the enzyme previously identified by isoelectric focusing of the purified RIL. In all cases, the Lac2 band shows higher intensity with respect to Lac1 which changed with the different inducers used. The higher activity in the slabs was obtained for Lac2 in the samples supplemented with phenylhydrazine, OMW, guaiacol, 4-methylcatechol and catecol. The other aromatic compounds gave lower stimulating effect on the RIL isoenzymes production (Fig. 4).

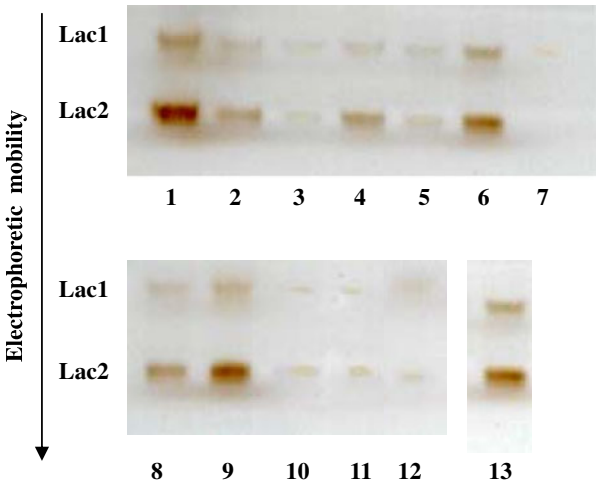

Fig. 4 Native PAGE of extracellular protein produced by *R. lignosus* in the presence of different inducers (1 mM). Zymograms of laccase isoenzymes Lac1 and Lac2 with activity staining. Lanes: *1*, phenylhydrazine; *2*, pirogallol; *3*,vanillic acid; *4*, catechol; *5*, ferulic acid; *6*, 4-methylcatechol; *7*, basal medium; *8*, hydroquinone; *9*, guaiacol; *10*, catechin; *11*, caffeic acid; *12*, gallic acid; *13*, OMW

Moreover, the intensity of the electrophoretic bands that indicate the level of laccase isoenzymes synthesis agrees with the enzymatic activity shown in Rl cultures supplemented with various inducers (Fig. 1). These results confirm previous studies carried out in *Phlebia radiata* [37] which found that aromatic compounds differentially affect the synthesis of laccase isoenzymes.

Laccase Induction by Olive Mill Wastewater

In previous studies, it has been reported that the laccase production ability can be increased several times on supplementing the basal growth media of different fungal strains with various natural materials such as cotton stalks [38], sugarcane bagasse, wheat straw and rice straw [39, 40] and molasses wastewater [41]. Moreover, agrochemical or industrial compounds also increased laccase production in liquid cultures of *Trametes versicolor* [42]. The results obtained by OMW demonstrated that this material shows a great capability to increase laccase production when added to the Rl strain growing on the basal medium. Laccase induction depended on the OMW proportion in the growth medium; highest enzyme activity (82 U ml^{-1}) was observed at the 21st day of cultivation in a medium at pH 4.2, containing 10% of OMW in water (*v*/*s*), corresponding to a phenolic concentration equal to 320 mg l^{-1} (Fig. 1). At higher OMW concentration (upper 10%), we found an inhibitory effect on the enzyme activity. These results are in agreement with previous studies showing that the maximum laccase activity from *Pleurotus ostreatus* was reached at phenolic concentrations below 0.5 g l^{-1} [43]. However, highest laccase activity has been reported in *Phanerochaete flavidoalba* at phenolic concentration of 2.8 g l^{-1} [44]. This effect could be due to reasons such as a different phenolic composition of OMW, greatly dependent on the extraction system, and the variety of the cultivars and/or the variability of fungal strains [45].

In conclusion, the overall results reported here indicated that the same natural and synthetic phenolic compounds could be considered as efficient inducers to enhance the laccase production in Rl culture.

Acknowledgements This work was supported by a grant of Istituto Nazionale Biostrutture e Biosistemi (I.N.B.B) and FIRB RBRNO7BMCT.

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