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The Role of Laccase from White Rot Fungi to Stress Conditions

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This study was performed in order to investigate the effect of selected xenobiotic compounds, such as different stress factors, Cd(Ⅱ), Zn(Ⅱ), Cu(Ⅱ) and Mn(Ⅱ) ions or some pro–oxidants: menadione, paraquat and hydrogen peroxide, on laccase activity with some white–rot fungi. Changes in laccase activity in the presence of natural fungicidals, the saponins from *Medicago sativa* roots, were also examined. The introduction of both metal ions and some pro–oxidants into the medium enhanced a multiple increase in extracellular laccase activity for the two fungal strains, *Trametes versicolor* and *Abortiporus biennis*. An notable increase in laccase level had already appeared in the 36 hour by the treatment with oxidative stress factors (menadion, paraquat and hydrogen peroxide), anti–fungal substance saponines as well as metal ions. The enhanced activity of laccase under heavy metal ions presence also suggests its important role in the fungal adaptation processes, what could be utilized to heavy metals containing waste materials. These strains of white–rot fungi could also be used to remove heavy metals from aqueous solutions by adsorbing the metals on mycelium and to biodegrade various organic xenobiotics by extracellular laccase. The presence of harmful compounds in the medium which might appear lethal for other organisms arouses detoxification abilities of this fungal group by increasing extracellular discharge of ligninolytic enzymes, in particular laccase. This offers extremely large possibilities for biotechnological use of white–rot fungi.

INTRODUCTION

During the whole development cycle living organisms are subject to different kind of stress conditions, such as changes in temperature, UV radiation or various xenobiotic compounds appearing in the environment (Jamieson, 1995; Koga and Takumi, 1995). Consequently they have developed during their evolution a range of effective enzymatic or non–enzymatic systems of response to stress factors (Mager and Kruijff, 1995; Ruis and Schiller, 1995). Basidiomycetes species, presented in this report, are considered to be a very interesting but little known group of fungi. Given their exceptional adjustment abilities, they can accommodate to detrimental conditions of the environment where they continue to act as natural lignocellulose destroyers (Tuor *et al*., 1995).

Our experiments imply a distinct influence that various stress factors exert upon the activity of laccase the oxidoreductase taking part in lignin depolymerization (Thurston, 1994; Collins and Dobson, 1997). This enzyme, belonging to polyphenolic oxidases, can be used in biotechnological processes, for instance whitening wood pulp, decomposition of different kinds of biopolymers or detoxification of environmental pollutants (Monteiro and Carvalho, 1998; D'Annibale *et al*., 1999). Laccase oxidizes a number of aromatic hydrogen donors forming free phenoxy radicals and catalyzes reactions of decarboxylation and demethylation of phenolic and methoxyphenolic acids (Thurston, 1994; Potthast *et al*., 1995). Laccases are produced by the majority of white–rot fungi described to date as well as by other types of fungi and by plants (Aramayo and Timberlake, 1990; Wahleithner *et al*., 1996). There are many reports concerning influence of a number of substances on the activity of extracellular laccase in various white–rot fungi. These data were collected in Table 1.

This experiment was carried out to compare the effect of selected xenobiotic compounds on laccase activity with some white–rot fungi. *Trametes versicolor* and *Abortiporus biennis* cultures were grown in liquid mineral media, and subjected to different stress factors such as Cd(Ⅱ), Zn(Ⅱ), Cu(Ⅱ) and Mn(Ⅱ) ions or some pro–oxidants: menadione, paraquat and hydrogen peroxide. Changes in laccase activity in the presence of natural fungicides, such as saponins from *Medicago sativa* roots, were also examined.

MATERIALS AND METHODS

Fungal material and culture conditions

White–rot basidiomycetes – *Abortiporus biennis* (Bull.ex Fr.) Sing and *Trametes versicolor* (L. ex Fr.) Pil were obtained from the culture collection of the Department of Biochemistry, Maria Curie–Sklodowska University of Lublin, Poland. Fungi were maintained on 2% (wt/vol) malt agar slants. For inoculation the fungal agar plugs (*ca.* 0.5 cm^2) were cut and grown in a

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Table 1. The induction of extracellular laccase in the culture of selected strains of Basidiomycetes

Strain	Compounds (concentration)	Increase of laccase activity to control cultures	References
Trametes versicolor	$CuSO_4$(400 μM)	18x	Collins *et al*., 1997
Trametes versicolor	NH_4+ (60 mM)	15x	Collins *et al*., 1997
Trametes versicolor	2,5–xylidine (500 μM)	40x	Collins *et al*., 1997
Trametes versicolor	1–hydroxybenzotriazole (500 μM)	25x	Collins *et al*., 1997
Phanerochaete chrysosporium	$CuSO_4$ (400 μM)	8x	Dittmer *et al*., 1997
Pycnoporus cinnabarinus	2,5–xylidyne (10 μM)	9x	Eggert *et al*., 1996
Pycnoporus cinnabarinus	lignosulfonate (1%)	3x	Eggert *et al*., 1996
Pycnoporus cinnabarinus	veratric alcohol (1 mM)	2x	Eggert *et al*., 1996
Pleurotus eryngii	alkalic lignin (400 μM)	6x	Munoz *et al*., 1997
Pleurotus eryngii	vanilic acid (400 μM)	4x	Munoz *et al*., 1997
Pleurotus eryngii	veratric acid (400 μM)	3x	Munoz *et al*., 1997

basal medium (Lindeberg, 1952) for *A. biennis* and for *T. versicolor* (Fahreus and Rainhammar, 1967). Culture medium contained glucose as the main source of carbon, L–asparagine as the main source of nitrogen and trace element's solution. The cultures were grown in static flasks at 25 °C till the mycelium colonized the whole surface of the liquid. The mycelial mats were collected and homogenized in a Warning blender. The stationary cultures, after inoculation with 4% (v/v) of the homogenate, were incubated at 25 °C in 25 ml Erlenmayer flasks containing 10 ml medium (Fahreus and Rainhammar, 1967). The extracellular medium was separated from mycelium by filtration through Miracloth (Calbiochem). All measurements were recorded using extracellular cultures of 5th day after addition of heavy metals, parquat, menadione, hydrogen peroxide and saponins.

Stress conditions

The 10–day–old cultures of *A. biennis* and *T. versicolor* were treated with different kinds of stress factors such as Cd(II), Cu(II), Zn(II) and Mn(II) ions, and to selected pro–oxidants: menadione, paraquat and hydrogen peroxide. Additionally, changes in laccase activity in the presence of natural fungicidals, the saponins *Medicago sativa* roots, were examined.

Laccase assays

Laccase activity was measured by monitoring the oxidation of syringaldazine (Leonowicz and Grzywnowicz, 1981). The reaction mixture contained 0.025 mM syringaldazine, 50 mM citrate–phosphate buffer pH 5.2 and enzyme. The oxidation of syringaldazine was monitored by the increase in the A_{520} (ε_{520} = $6.5\times10^4 M^{-1}cm^{-1}$).

Determination of protein

Protein concentrations were determined by using Bradford reagent and bovine serum albumin as the standard (Bradford, 1976).

RESULTS AND DISCUSSION

Our investigations showed the introduction of both metal ions and some pro–oxidants into the medium to enhance a multiple increase in extracellular laccase activity for the two fungal strains. An notable increase in laccase level had already appeared in the 36th hour following the treatment with oxidative stress factors (menadion, paraquat and hydrogen peroxide), anti–fungal substance saponines as well as metal ions (Tables 2 and 3). The increased laccase level initiated by high temperature was described by Fink–Boots *et al.* (1999) in the cultures of selected strains of Basidiomycetes as the consequence of adaptation to changes of environmental temperature conditions. The enhanced activity of laccase under heavy metal ions presence (Table 2) also suggests its important role in the fungal adaptation processes, what could be utilized to heavy metals containing waste materials.

These strains of white–rot fungi could be used to remove heavy metals from aqueous solutions by adsorbing the metals on mycelium and to biodegrade various

Table 2. The influence of various divalent cations on the activity of extracellular laccase in the cultures of *Abortiporus biennis* and *Trametes versicolor*

Heavy metals	*Abortiporus biennis*	*Trametes versicolor*
Cd (II)	14.5 ± 0.8	5.8 ± 1.2
Zn (II)	3.8 ± 0.9	8.2 ± 1.4
Cu (II)	6.7 ± 1.2	7.4 ± 0.8
Mn (II)	2.2 ± 0.5	2.1 ± 0.4

Heavy metals were added to 10–day–old cultures. Data present activities of laccase from metal ions – amended cultures in comparison to control cultures. The laccase activities in control cultures were taken as 1.

Table 3. The influence of various inducers on the activity of extracellular laccase in the cultures of *Abortiporus biennis* and *Trametes versicolor*

Type of substance	*Abortiporus biennis*	*Trametes versicolor*
menadione	8.5 ± 0.7	10.2 ± 1.7
paraquat	4.9 ± 0.5	7.9 ± 1.2
hydrogen peroxide	6.1 ± 0.8	3.9 ± 0.4
saponins	4.2 ± 0.5	12.1 ± 1.8

Substances were added to 10–day–old cultures. Data present activities of laccase from substances – amended.

organic xenobiotics by extracellular laccase. This enzyme reduces oxygen to water and stimultaneously performs an electron oxidation of many aromatic substrates (polyphenols, methoxy–substituted monophenols, and aromatic amines) but in the presence of mediators, the substrate range of laccase can be extended (Burbonnaise *et al*., 1997). The different degree of degradation activity of white–rot fungi with respect to lignin and other organic compounds depends on the environmental conditions and the fungal species. Such an increased activity of laccase entails participation of the enzyme in response mechanism to external stress factors. This experiments have confirmed an extremely strong resistance of white–rot fungi to detrimental changes in the external environment. The presence of harmful compounds in the medium which might appear lethal for other organisms arouses detoxification abilities of this fungal group by increasing extracellular discharge of ligninolytic enzymes, in this case laccase. Even though our research does not conclusively explain the molecular aspects of laccase activity as a responce to various stress factors, the above sequence of reactions justifies describing this enzyme as important in fungal cell protection against stress. This offers extremely large possibilities for biotechnological use of white–rot fungi.

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