MONITORING OF LACCASE PRODUCTION BY FUNGAL ISOLATES FROM CZECH FOREST

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Abstract: Discovery of novel laccases with different substrate specificities produced by different fungal species is important for industrial, biotechnological and environmental applications. The aim of this work was to monitoring of laccase production by thirty five locally isolated white-rot fungal species. In five strains with the highest enzyme secretion laccase activity was examined using two different substrates (ABTS and syringaldazine) under different conditions (shaking, static). The measuring laccase activity using ABTS and syringaldazine as substrates confirmed that one of the best producers proved to be *Fomes fomentarius* and *Trametes* strains.

Key Words: white-rot fungi, ligninolytic enzyme, enzyme activity, laccase

INTRODUCTION

The white-rot fungi are able to degrade recalcitrant biopolymers such as lignin, wide range of pollutants and huge variety of materials (different type of wood, textile, plastic and many xenobiotics) due to their extracellular non-specific ligninolytic enzyme system. (Songulashvili et al. 2006, Tišma et al. 2010). Laccase (Lac, E.C. 1.10.3.2) and three heme peroxidases: lignin peroxidase (LiP, E.C.1.11.1.14), Mn dependant peroxidase (MnP, E.C. 1.11.1.13) and versatile peroxidase (VP, E.C. 1.11.1.16) are one of the most important ligninolytic enzymes, which are secreted extracelullary as secondary metabolites of different fungi. Their production is influenced by different aspects, such as fungal species, aeration (stationary or shaking) or time of cultivation (Elisashvili, Kachlishvili 2009).

The most important enzyme is laccase, which belongs to a family of blue multicopper oxidases containing four copper atoms per molecule in their catalytic center and catalyzes the four electron reduction of oxygen to water (Giardina et al. 2010). Laccase oxidizes many organic or inorganic compounds, including phenols and aromatic amines.

Laccase production by fungi is strongly affected by many fermentation parameters such as time of cultivation, stationary or submerged cultures, aeration by shaking or static conditions (Kocyigit et al. 2012).

The low substrate specificity makes this enzyme interesting for biotechnological purposes in various industries such as food, textile and for various technological applications, decolorization dyes, degradation of polyaromatic hydrocarbons and in nanobiotechnology as biosensors.

The use of enzyme for these purposes entails certain limitations. These are mainly the high-cost of commercial preparations and therefore are constantly looking for new, cheaper and natural sources of enzyme and the search for the most potential enzyme producers attains considerable attention (Songulashvili et al. 2006).

A number of screening studies of white-rot fungi are conducted to discover promising producers of ligninolytic enzymes (Kiiskinen et al. 2004, Songulashvili et al. 2006, Elisashvili, Kachlishvili 2009).

In recent years, new potential laccase producers such as marine fungi and different genera of basidiomycetes were found (Valeriano et al. 2009). Laccase-producing fungi are tested on solid media containing colored indicator compounds that facilitate the visual detection of laccase production or by liquid cultivations during which enzyme activity is monitored. Kiiskinen et al. (2004) screened novel

laccase producing fungi by a plate method based on polymeric dye compounds – guaiacol and tannic acid. The use of colored indicators is generally simpler as no sample handling and measurement are required. The screening strategy must aim to identify fungal strains and enzymes that will work under industrial conditions.

Therefore, the current study examined the laccase production and activity by different white-rot fungi from Czech forest to search new potential sources of laccase.

MATERIAL AND METHODS

Fungal strains and culture conditions

Thirty five locally isolated fungal strains (*Armillaria cepistipes*, *Armillaria ostoyae*, *Cerrena unicolor*, *Daedaleopsis confragosa*, *Fomes fomentarius*, *Ganoderma applanatum*, *Ganoderma carnosum*, *Ganoderma resinaceum*, *Grifola frondosa*, *Heterobasidion annosum*, *Inonotus dryadeus*, *Perenniporia fraxinea*, *Phellinus hartigii*, *Phellinus igniarius*, *Phellinus punctatus*, *Phellinus robustus*, *Phellinus tuberculosus*, *Pleurotus ostreatus*, *Schizophyllum commune*, *Stereum hirsutum*, *Trametes cervina*, *Trametes gibbosa*, *Trametes hirsuta*, *Trametes suaveolens*, *Trametes versicolor*) obtained from the Culture Collection of the Faculty of Forestry and Wood Technology of the Mendel University in Brno (Czech republic) were used in this study.

All strains were microscopically identified and kept on potato dextrose agar (PDA) at 4°C and periodically sub-cultured to maintain viability. All strains were tested for laccase production.

Cultures were cultivated on PDA for 10 days at 22°C. After this time three 1x1 cm^2 plugs were cut and added into Erlenmeyer flasks containing 40 ml of the Potato Dextrose Broth (PDB). The flasks were prepared in duplicates, first were incubated statically (28°C) and second were incubated in a shaker (150 rpm, 28°C). Supernatant was separated from the mycelia by centrifugation (10 000 rpm, 4°C, 5 min) and laccase activity was determined.

Qualitative laccase activity assay

The plugs of mycelium from each strain were inoculated onto PDA plates containing 0.3 g ABTS/300 ml PDA and then incubated at 28°C for 7 days. The formation of dark-green halo in the ABTS supplemented plates indicates a positive laccase secretion.

Enzyme assay

The enzyme activity was determined spectrophotometrically using a UV/VIS Lambda 25 Spectrophotometer (Perkin-Elmer). Laccase activity was measured at 415 nm by detecting the oxidation of 2,2-azino-bis-[3-ethyltiazoline-6-sulfonate] (ABTS, Sigma Aldrich) at pH 4.5 in 0.1M sodium acetate buffer (Bourbonnais, Paice 1990). For comparison, laccase activity was assayed by detecting the oxidation of syringaldazine (Sigma Aldrich) at 530 nm in 0.1M citrate phosphate buffer (Harkin, Obst 1973). One unit of enzyme activity was defined as 1 μmol of substrate oxidized per minute under the assay conditions. The enzyme activity assay was always performed in triplicate.

RESULTS AND DISCUSSION

Qualitative laccase activity assay

Preliminary screening of thirty five white-rot fungi for laccase production was carried out at the Petri dishes containing ABTS as indicator. Colored indicators are used for the visual recognition of laccase production (Gnanasalomi, Gnanadoss 2013).

Green color around the colonies was due to the oxidative polymerization of ABTS in the presence of extracellular fungal laccase. Among the 35 isolates 20 were found to be laccase positive by the formation of dark-green halo in ABTS supplemented plates and was considered as a positive reaction for laccase activity. The test confirmed that one of the best producers proved to be *Trametes* strains. Five fungi (*Trametes versicolor*, *Trametes gibbosa*, *Trametes suaveolens*, *Daedaleopsis confragosa*, *Fomes fomentarius*) (see Figure 1) were chosen for another experiments due to the highest color intensity. The isolates which did not show any color change were not considered for further work.

Our results agree with work Fonseca et al. (2010), who showed that *Trametes versicolor* had the highest color intensity of oxidation zones.

Figure 1 Fungi cultivated on Potato-Dextrose Agar with ABTS for 7 days

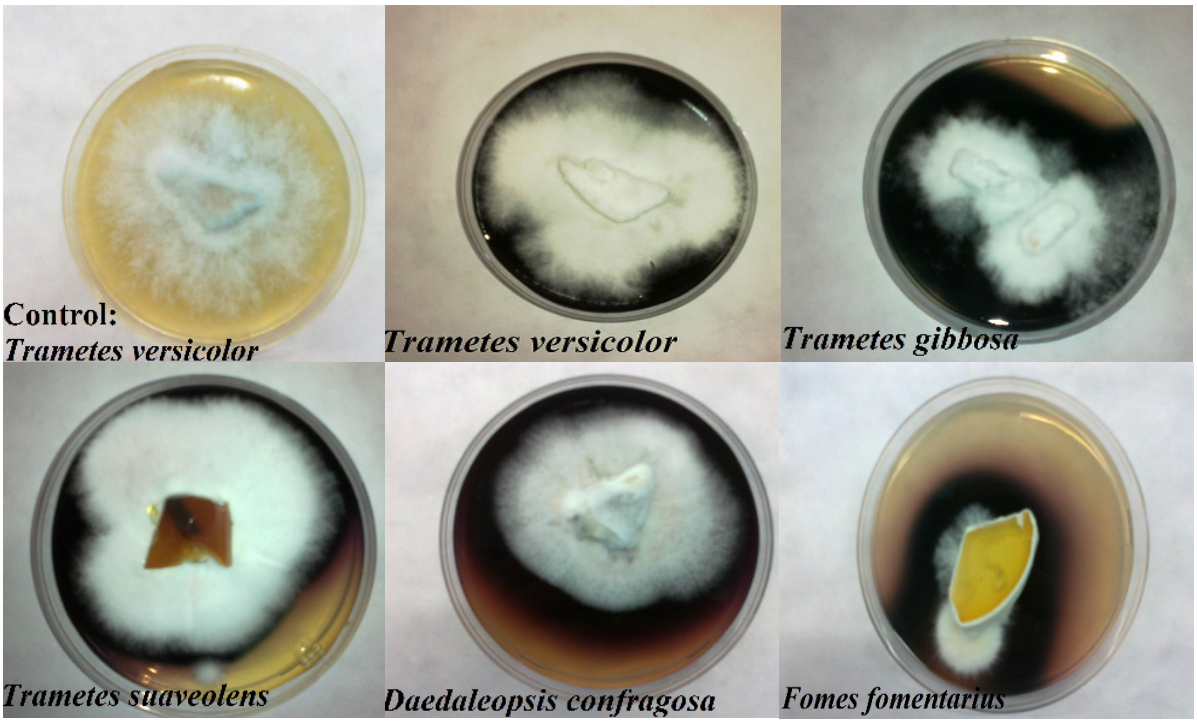
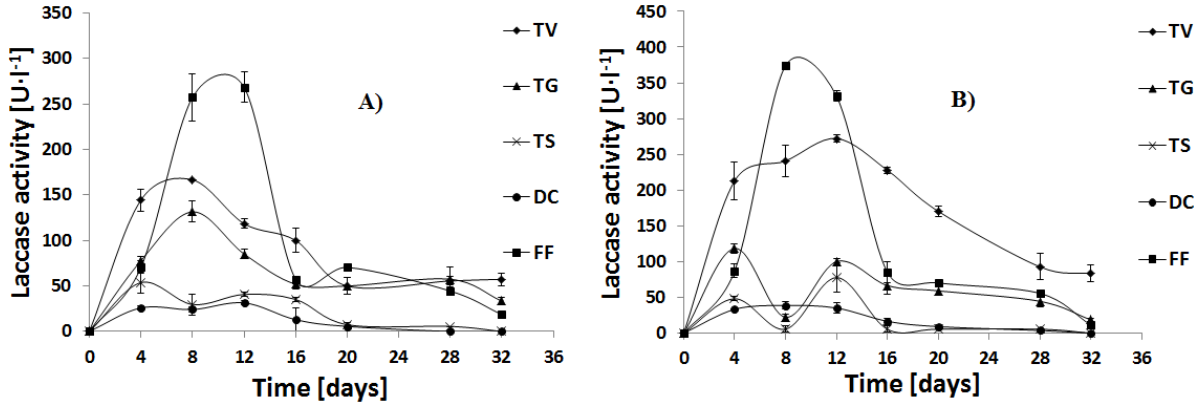


Figure 2 Laccase activity using ABTS as substrate A) Static conditions B) Shaking conditions



Legend: TV- Trametes versicolor, TG- Trametes gibbosa, TS- Trametes suaveolens, DC- Daedaleopsis confragosa, FF- Fomes fomentarius

Laccase activity

The enzyme production is species-dependent and strain-dependent and thus laccase secretion by new fungal strains from Czech forests has been studied to find new more important enzyme producers.

The laccase activity of five fungal strains *Trametes versicolor*, *Trametes gibbosa*, *Trametes suaveolens*, *Daedaleopsis confragosa* and *Fomes fomentarius* was studied using two different substrates (ABTS and syringaldazine) under different conditions (shaking, static).

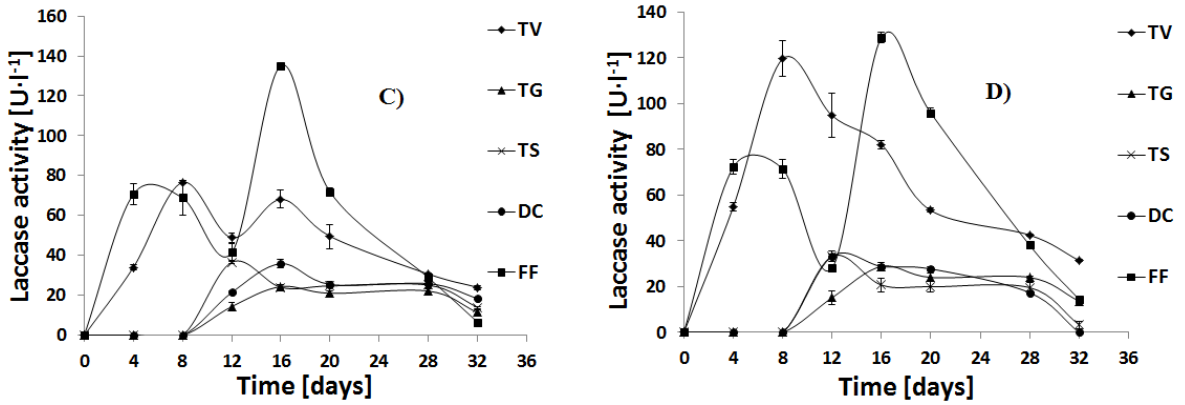
Trametes is known as a significant producer of ligninolytic enzymes. Therefore, many studies with *Trametes* strains have been extensively conducted. Our results indicated that *Trametes sp.* and *Fomes fomentarius* seem to be one of the best producers of laccase under shaking conditions. Our results agree with the study of Songulashvili et al. (2007), where *Fomes fometnarius* was observed as the best producer of laccase using ABTS and syringaldazine as substrates. Opposite results were

published in the work of Rodrigues et al. (2008), where *Trametes versicolor* was better laccase producer in comparison with *Fomes fomentarius*.

In relation to the influence of different culture conditions (static or shaking) on laccase activity, our results are comparable to those described by other authors (Kocyigit et al. 2012, Dong et al. 2005), who have reported increases of enzyme activity under shaking conditions (see Figure 2A–B).

The literature data reporting laccase activity of white-rot fungi are usually based on the use of nonspecific substrates like ABTS or naphtol while syringaldazine oxidation has rarely been reported. Cordi et al. (2007) studied laccase activity of *Trametes versicolor* and the reagent syringaldazine was used as substrate. The results agree with our data under static conditions (see Figure 3C–D). Similar results were observed in the work of Minussi et al. (2007), who detected laccase activity using syringaldazine as substrate in a liquid culture and in their study the maximum value for laccase activity was obtained with *Trametes versicolor* at 21 days of growth.

Figure 3 Laccase activity using syringaldazine as substrate C) Static conditions D) Shaking conditions



Legend: TV- Trametes versicolor, TG- Trametes gibbosa, TS- Trametes suaveolens, DC- Daedaleopsis confragosa, FF- Fomes fomentarius

CONCLUSION

The screening study revealed five white-rot fungi as suitable laccase producers. The measuring of laccase activity confirmed that one of the best producers seems to be *Trametes versicolor* and *Fomes fomentarius*. It seems to be preferable to use a substrate ABTS than syringaldazine.

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