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Comparative Studies of Extracellular Fungal Laccases under Different Conditions

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ABSTRACT

Various basidiomycetes and deuteromycetes, grown in liquid and solid culture media, were compared for their laccase-producing ability and for the inducing effect of soil and agricultural residues on laccase production. Laccase activity in extracts of all solid media was higher than that of the liquid media. In liquid cultures, fungal laccase activity in extracts of pea (*Pisum sativum*) straw-treated media compared with the other agricultural residue-treated media was significantly low. The laccase-producing ability of *Polyporus* sp. compared with the other fungi was significantly high whereas that of *Trichoderma reesei* was markedly low. Laccase activity of *Phanerochaete chrysosporium* under liquid conditions was strikingly low, however, under solid conditions it increased more than that of the other fungi. With the addition of soil to wheat and rice straw-treated solid media, fungal laccase activity increased significantly.

Keywords: Agricultural residue, Fungi, Laccase activity, Liquid medium, Solid medium.

INTRODUCTION

Laccases (benzendiol; oxygen oxidoreductase EC1.10.3.2) are polyphenol oxidases common in plants and white-rot basidiomycetes that were first isolated from the latex of the Japanese *Rhus vernicifera* tree in 1883. They are extracellular copper-containing oxidases, which constitute one of the most important ligninolytic enzymes. Laccase catches one electron from phenols although, in the presence of appropriate substrates, it is able to promote Cα-Cβ cleavage of non-phenolic β-1 model compounds and to oxidize veratryl alcohol [6, 17, 22, 28]. It is also able to oxidize aromatic pollutants (*ortho*-, *meta*- and *para*-substituted phenols), such as anilines, chloro-phenols, benzopyrenes and, particularly, phenolic dyes in the presence of oxygen [4, 21, 28 , 30].

In this reaction, the substrates are oxidized by one electron to generate the corresponding phenoxy radicals, which either polymerize to yield a phenolic polymer or are further oxidized by laccase to produce a quinone. Electrons received from the substrate are subsequently transferred to oxygen, which is reduced to water [30, 28]. In addition to polymerization, which amounts to a cross-linking of lignin via oxidative coupling, this enzyme also catalyzes another important reaction in lignin. It hydroxylates phenolic substrate, thereby introducing phenolic hydroxyl group that serves as a new reactive site on the molecule [27]. These catalytic properties make the enzyme an interesting candidate for a variety of possible uses in lignin biotranformation (especially in the wood and plastics industries), and in aromatic pollutant decontamination [4, 11, 19].

Laccases also have several different physiological functions [3, 4, 11]. The detoxification of different aromatic compounds and the defense mechanism of laccase-

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producing fungi against potential biocontrol fungi have been suggested as some of the roles for laccases of fungi [12, 13, 18].

Peroxidases and/or phenoloxidases can act on specific recalcitrant pollutants by precipitation or transforming them into other products and permitting a better final treatment of the waste [6]. Nevertheless, despite the importance of laccase in such processes, most studies on this enzyme deal with its purification and characterization instead of its production. Therefore, considering the industrial and environmental importance of laccase, studies to enhance laccase-producing ability of the laccase-producing organisms are required [16].

To obtain more information about the laccase activity of fungi in various environments, the laccase-producing ability of *Trichoderma reesei, Aspergillus terreus, Phanerochaete chrysosporium, Armillaria* sp. and *Polyporus* sp., as well as the inducing effect of soil and agricultural residues on laccase production in liquid and solid media have been studied. The main objective of the present study was to compare the laccase-producing ability of fungi in different conditions.

MATERIALS AND METHODS

Microorganisms

Fungi, including the Deuteromycetes, *A. terreus* and *T. reesei*, and the Basidiomycetes, *Armillaria* sp., *Polyporus* sp., and *P. chrysosporium*, were obtained from the Microbiology Laboratory of the Science Faculty of Isfahan University. All of them were native isolates except *P. chrysosporium* which had been prepared by CMI, Hswaisi Co. in Russia.

Media and Culture Conditions

All organisms were cultured and maintained (at 4 ^{o}C) on commercially prepared Potato Dextrose Agar (PDA) from Merck Co. liquid media containing 1.0 g of milled agricultural residues (<2 mm) in 100 mL of distilled water and were prepared in 250-mL Erlenmeyer flasks. The agricultural residues used were wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rice (*Oryza sativa*), pea (*Pisum sativum*) straw and wood shavings. These were air-dried, ground (<2mm) and autoclaved at 121 ^{o}C for 15 minutes. The Avicel liquid culture medium contained insoluble microcrystalline cellulose 10 g, proteus pepton 0.5 g, urea 0.3 g, Tween-80 0.2 g, $(NH_4)_2SO_4$ 1.4 g, $MgSO_4.7H_2O$ 1 g, K_2HPO_4 1 g, $CaCl_2$ 0.3 g, KH_2PO_4 0.2 g, $FeSO_4.7H_2O$ 0.005 g, $CoCl_2$ 0.002 g, $MnSO_4$ 0.0016 g, and $ZnSO_4.7H_2O$ 0.0014 g in 1000 mL of distilled water (with a starting pH of 5).

Solid media were prepared by adding 1.0 g of milled agricultural residues (including wheat, barley and rice straws) to 5 mL of distilled water in 250-mL Erlenmeyer flasks [29]. For the study of soil effects, two soil treated solid media were prepared by adding 25.0 g of autoclaved soil and 1.0 g of milled agricultural residues (wheat or rice straws) to 15 mL of distilled water. The soil was classified as Xerollic Camborthids, fine-carbonatic, thermic, and sampled from the upper trace of the Zayeanderude River in Isfahan. The soil sample was air-dried and then crushed and sieved to pass a 2-mm mesh screen before physical and chemical analysis. Soil pH was equal to 7.7 and the soil texture was clay loam. The soil had 42% equivalent $CaCO_3$, 1.5% organic matter, 20.4 cmolc kg^{-1} cation exchange capacity, and 1.28 dS m^{-1} electrical conductivity. Soil particles smaller than 2 mm were autoclaved at 121 ^{o}C for 30 min before being added to the culture media.

All liquid and solid media prepared were autoclaved at 121 ^{o}C for 15 minutes. Soil-treated media were autoclaved at 121 ^{o}C for 30 minutes. After cooling, the vegetative mycelia of fungi from PDA plates (two 0.25 cm^2 agar plugs) were inoculated in 250-mL Erlenmeyer flasks. The liquid media were agitated (120 rev min^{-1}) for aeration. Inocu-

lated cultures were incubated at 28 ^{o}C. Each treatment was carried out in 3 replicates.

Enzyme Preparations and Assays

At different intervals over a three-week period, extracts of liquid media were obtained using Whatman No.1 filter paper. The pH of the extracts was measured. Extracellular laccases of solid media in 250-mL Erlenmeyer flasks were extracted by 100 mL of 25 mM sodium acetate (pH 5.5) after 1.5 hours shaking.

Laccase activity in the extracts was assessed in a 1.0 mL reaction mixture containing 75 mM catechol as a substrate, a 50 mM sodium phosphate buffer (pH 5.0), and 0.2 mL culture extracts [22]. The progress of the reaction was monitored at 440 nm for 10 minutes. One unit of laccase activity was defined as a change of A_{440} (ΔA_{440}=1) in a 1.0 mL sample within 1.0 minute.

Means were calculated and Duncan's new multiple range test was conducted to assess laccase activities under different conditions.

RESULTS AND DISCUSSION

The mean pH of the liquid cultures during 3 weeks of incubation is shown in Figure 1. The pHs of the liquid cultures, ranked in descending order, were as follows: pea> wood> rice> barley> wheat> Avicel. Culture pH was considered as an index of fungal growth and activity. When fungal activity was high, the pH value was low and *vice versa*. Easily degradable organic matters were more suitable for fungi, so the pHs of these cultures (except pea straw) were lower than the pHs of wood and rice cultures [25]. In addition, the pH of easily degradable organic matter cultures was decreased more by the Deuteromycetes *A. terreus* and *T. reesei*. However, the pH of wood and rice cultures was decreased efficiently by the Basidiomycetes, especially *Polyporus* sp..

The means of laccase activities in the extracts of liquid media treated with different substrates are given in Table 1. Laccase activities in Avicel and wheat treated media were relatively high due to the optimal growth conditions. Although barley straw

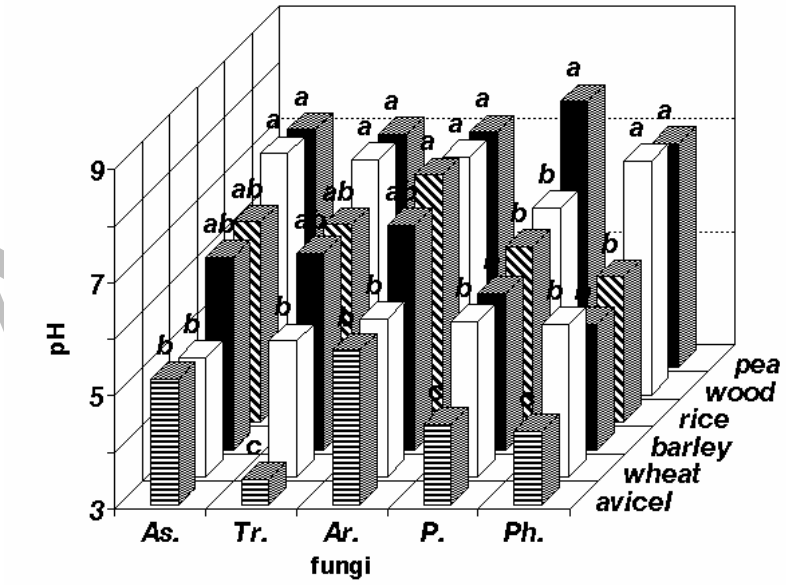

Figure 1. Average pH of the culture extracts inoculated by *As.- A.terreus, Tr.- T.reesei, Ar.- Armillaria* sp., *P.-Polyporus* sp., and *Ph.- P.chrysosporium* (Means followed by the same letter are not significantly different ($p<0.05$)).

was more suitable than rice for fungi, the laccase activities of fungi were relatively high in both rice and wood cultures. Laccase activities in pea treated media were negligible. These results show that laccase production is highly dependent on the culture media used and/or may be induced by its substrates. The stimulation of laccase by the addition of lignocellulosic wastes into the culture media has been also reported [26 16].

The laccase activities of the Basidiomycetes, namely *Polyporus* sp., were significantly higher than that of the other fungi (Table 2). The order of the fungi tested according to their laccase-producing ability in liquid media was: *Polyporus* sp. > *Armillaria* sp.> *A. terreus* = *P. chrysosporium*> *T. reesei*. Strikingly, laccase activities of *P. chrysosporium* were relatively low in the liquid culture media. This fungus has been extensively studied as a model organism for fungal lignin degradation. It is widely described as a white-rot fungus lacking laccase activity, but able to degrade lignin very well [5, 9, 10, 15].

Laccase activity in all the extracts of solid media was higher than those of the liquid media. Laccase activity in the extracts of solid media decreased in the following order: rice + soil, wheat + soil, rice, wheat, and barley (Table 3). Although the addition of soil to the solid media of *Polyporus* sp. did not increase laccase activities significantly, the addition of soil significantly increased laccase activities of fungi. Ardon *et al*. [2] reported that cotton–stalk extract (CSE) stimulates both fungal growth in surface cultures and extracellular laccase activity in submerged cultures. It was reported that a flavonoid plays an important role in the transcription and translation of the enzyme laccase. Induction of laccase activity was de-

Table 1. Average laccase activities of fungi in extracts of the liquid media treated with different substrates.[a]

Culture media	Mean[b]	95% Confidence interval	
		Lower	Upper
Avicel	0.0264 [ab]	0.010292	0.042508
Wheat	0.0336 [a]	0.014655	0.052545
Barley	0.0126 [bc]	0	0.034135
Rice	0.012 [bc]	0	0.036724
Wood	0.015 [bc]	0	0.041442
Pea	0 [c]	0	0.004404

[a] One unit of laccase activity was defined as a change in A_{440} (ΔA_{440}=1) of 1.0 mL sample within 1.0 min.
[b] Means followed by the same letter are not significantly different ($p<0.05$).

Table 2. Average laccase activities of different fungi in extracts of the liquid media.[a]

Fungi	Mean[b]	95% Confidence interval	
		Lower	Upper
A. terreus	0.010767 [bc]	0	0.028512
T. reesei	0.006233 [c]	0	0.016644
Armillaria sp.	0.018333 [b]	0.007274	0.029393
Polyporus sp.	0.038167 [a]	0.014435	0.061898
P. chrysosporium	0.0095 [bc]	0	0.022485

[a] One unit of laccase activity was defined as a change in A_{440} (ΔA_{440}=1) of 1.0 mL sample within 1.0 min.
[b] Means followed by the same letter are not significantly different ($p<0.05$).

Table 3. Average laccase activities of fungi in extracts of the solid media treated with different substrates.[a]

Culture media	Mean[b]	95% Confidence interval	
		Lower	Upper
Wheat	0.0278 [c]	0	0.07862
Barley	0.0232 [c]	0	0.070231
Rice	0.034 [bc]	0	0.113597
Wheat + soil	0.0632 [ab]	0.03793	0.08847
Rice + soil	0.0744 [a]	0.042518	0.106282

[a] One unit of laccase activity was defined as a change in A_{440} (ΔA_{440}=1) of 1.0 mL sample within 1.0 min.
[b] Means followed by the same letter are not significantly different ($p<0.05$).

pendent upon the concentration of the supplemented extract [1]. So, the higher laccase activity in the both solid media and soil treated solid media may be related to the higher concentration of these stimulants.

As observed in liquid media, laccase activities of the Basidiomycetes (especially *Polyporus* sp.) in extracts of solid media were markedly higher than those of the Deuteromycetes (Table 4). These differences in solid media were more obvious than in liquid media. Surprisingly, laccase activity of *P. chrysosporium* in solid media increased and was significantly higher than that of the Deuteromycetes. The presence of lignin peroxidases (LiPs) and manganese peroxidases (MnPs) in *P. chrysosporium* is well established, but *P. chrysosporium* is widely quoted as an example of a white-rot fungus that does not produce laccase [5,10,15]. It was not known whether the inability to demonstrate the presence of laccase in *P. chrysosporium* cultures was due to the use of culture conditions which are not favorable for laccase production by this organism or that the organism lacks the genetic machinery for producing laccase. However, a study on the regulation of expression of LiP and MnP genes in cellulose- and wood-grown cultures of *P. chrysosporium*, indicated that this fungus produces low but consistent levels of laccase [26]. Srinivasan *et al.* [26] presented evidence for the presence of laccase in *P. chrysosporium* grown in low-nitrogen (2.4 mM) or high-nitrogen (24 mM) defined media containing cellulose as the carbon source.

Table 4. Average laccase activities of different fungi in extracts of the solid media.[a]

Fungi	Mean[b]	95% Confidence interval	
		Lower	Upper
A. terreus	0.0258 [cd]	0	0.064455
T. reesei	0.0192 [d]	0	0.051428
Armillaria sp.	0.0324 [bc]	0	0.074338
Polyporus sp.	0.1092 [a]	0.07993	0.13847
P. chrysosporium	0.036 [b]	0.000477	0.071523

[a] One unit of laccase activity was defined as a change in A_{440} (ΔA_{440}=1) of 1.0 mL sample within 1.0 min.
[b] Means followed by the same letter are not significantly different ($p<0.05$).

CONCLUSION

This study revealed that laccase activities by the Basidiomycetes especially -*Polyporus* sp. - were considerably higher when compared with those of the Deuteromycetes. Although the laccase-producing ability of *P. chrysosporium*, (one of the most efficient ligninolytic microorganisms known to date) was strikingly low under liquid conditions compared to the other fungi, laccase activity of *P. chrysosporium* in solid media was, surprisingly, significantly higher than that of the Deuteromycetes.

It has been reported that cotton straw contains substances that stimulate lignin degradation [20, 1]. Platt *et al*. [20] found that *Pleurotus ostreatus* degrades cotton straw faster than wheat straw, but the degradation of wheat straw is accelerated by the addition of an aqueous extract of cotton straw. It was reported that a flavonoid component present in the cotton extract could be responsible for the improved lignin degradation [1]. In the present study, the laccase activity of all fungi in Avicel liquid medium was relatively high. This may be related to the stimulation of laccase production by the higher concentration of cellulose derivatives in this medium. In liquid cultures treated with wheat straw laccase, activity was significantly high. This result may be related to the stimulation of laccase production by both cellulose and flavonoid components. This conclusion needs to be studied further.

Results indicated that, among the agricultural residues used in different conditions, the addition of rice straw to the solid media had a remarkable effect on the production of laccase. It was reported that Basidiomycetes produce more ligninolytic enzyme under stressful conditions and C, N, and S starvation [4, 8, 11, 19]. These conditions and a higher concentration of stimulants may be the cause of that result.

Generally, fungal laccase activities in extracts of solid media were higher when compared with those in extracts of liquid media. This result may be related to i) lower deactivation of enzymes after adsorption or immobilization on agricultural residues in solid media [6, 14, 24], and/or ii) more stimulation and production of enzymes in solid media. The addition of soil to the solid media increases laccase activity significantly. It is perhaps related to soil organic matter, especially aromatics [23], hydrocarbons [26], flavonoids [1], and trace elements [19]. So, the induction effects of soil components (soil solids or soil extracts) on laccase production by fungi and the interaction between soil aromatic compounds and catechol during laccase assays [27], need to be studied further in future.

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مقایسه فعالیت لاکاز برون یاخته‌ای قارچ ها در شرایط مختلف

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چکیده

توان ساخت آنزیم لاکاز قارچ ها و تاثیر خاک و مانده‌های کشاورزی در برانگیختن ساخت این آنزیم با کشت انواعی از بازیدیومیست‌ها و قارچ های ناقص در محیط‌های کشت مایع و جامد بررسی شد. فعالیت آنزیم لاکاز در عصاره محیط های کشت جامد بالاتر از محیط‌های مایع بود. فعالیت آنزیم لاکاز قارچ ها در کشت های مایع دارای کاه نخود در برابر کشت‌های دیگر مانده‌های کشاورزی به اندازه چشم گیری پایینتر بود. توان قارچ *پلی پوروس* در ساخت آنزیم لاکاز به اندازه چشم‌گیری بیشتر از قارچ های دیگر بود، در برابر آن قارچ *تریکودرما ریسی* توان اندکی از خود نشان داد. فعالیت آنزیم لاکاز قارچ *فانروکت کریزوسپوریوم* در محیط‌های کشت مایع بسیار اندک بود ولی در محیط‌های جامد فعالیت آن تا حدی بیشتر از قارچ‌های دیگر افزایش داشت. با افزودن خاک به محیط‌های کشت جامد کاه برنج و گندم فعالیت آنزیم لاکاز قارچ‌ها به اندازه چشم‌گیری افزایش یافت.