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Appearance of Laccase in Wood-Rotting Fungi and Its Inducibility*1

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목재부후균으로부터 Laccase 효소의 생산 및 유도

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요 약

목재부후균으로 부터 락케이스 효소의 생산 및 유도를 위하여 여러가지 유도약품(inducer)을 사용하였다. 이들 가운데 ferulic acid, pentachlorophenol 및 2,5-xylidine이 매우 높은 락케이스 활성을 나타나게하였으며, 거의 동일한 유도효과를 보여주었다. 이들 약품 이외에도 sinapic acid, syringic acid 및 coffeic acid 등도 높은 락케이스 활성을 주었는데, 산의 형태가 알데히드류보다도 높은 유도효과를 나타냈다. 그리고 실험한 48개 균주가운데 38개 균주가 락케이스를 생산하였으며, 이 가운데 32균주가 ferulic acid에 의해 강한 효소유도 활성을 보였다. 이러한 결과는 지금까지 락케이스 효소의 검출이 어려웠던 *Abortiporus biennis* 및 *Gleophyllum odoratum*에서도 높은 락케이스 효소의 유도를 가능하게 하였다. 아울러 가장 높은 효소활성을 나타낸 균주로서는 *Cerrena unicolor* 였으며, 그 락케이스 효소활성이 무처리 및 inducer 첨가시 각각 40,000 및 60,000 nkat/l 정도 였다.

Keywords : Laccase, ferulic acid, inducer, white-rotting fungi, *Cerrena unicolor*, constitutive enzyme, 2,5-xylidine pentachlorophenol

1. INTRODUCTION

Fungal laccase seems to be a promising enzymic agent applicable for various biotechnological process. Laccase and a very similar polyphenol oxidase can be used as a free enzyme and an immobilized preparation both in water and in some organic solvents, improving in several processes this way (Mil-

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stein *et al.*, 1993; Burton & Duncan, 1995). Of possible applications, the enzyme is considered to be a bleaching agent in the pulp and paper technology (Bourbonnais *et al.*, 1995), a stabilizer during the must and wine processing or as a dechlorinating factor. Its broad activity on hydrogen donors provides opportunities for detoxification of some terrestrial pollutants (Bollag *et al.*, 1986) as well as for removing certain phenolics and other aromatic compounds from natural and industrial wastewater (Nannipieri & Bollag, 1991).

Laccase produced by several basidiomycete, ascomycete and deuteromycete fungi. The enzyme is induced mainly by 2,5-xylidine. This compound was described for the first time by Fahraeus *et al.*(1949) in the case of fungus *Coriolus versicolor* (synonym of *Trametes versicolor*). They selected an inducer from various isomers of 2,5-xylidine and found over 160-fold stimulation of laccase when the compound was introduced into the growing culture. It was shown further that not only in *Coriolus versicolor* but also in other lignin degrading fungi like *Fomes annosum*, *Pholiota mutabilis*, *Pleurotus ostreatus* (Agematu *et al.*, 1993) and *Phlebia radiata* (Rogalski & Leonowicz, 1992), laccase can be induced by 2,5-xylidine. Also some phenolic compounds stimulate laccase production effectively, e.g. ferulic acid which considerably induces the enzyme in the cultures of *Pholiota mutabilis, Pleurotus ostreatus* and *Trametes versicolor*(Leonowicz & Trojanowski, 1975a; Leonowicz & Trojanowski, 1975b; Leonowicz *et al.*, 1978; Leonowicz & Grzywnowicz, 1981). Unfortunately, the fungal sources of laccase are not suitable for biotechnology, because of application of the compounds toxic for the environment, like phenolics or xylidine which are necessary for its induction.

Non-induced cultures usually produce rather small amounts of laccase, not sufficient for preparation on a commercial scale. Looking for the better sources of non-induced laccase, this paper presents the results of our screening of fourty eight wood rotting terrestrial fungi for the enzyme production and its inducibility.

2. MATERIALS & METHODS

All fungal strains tested were from the culture collections of the Department of Biochemistry, University of Lublin, Poland (FCL) and as well as Department of Forest Products, Chungbuk National University, Cheongju, Korea (FCC). The fungi tested were as follows: *Abortiporus biennis* (Bull. ex Fr.) Sing., *Agaricus arvensis* Schaeff ex Fr., *Agaricus bisporus* (Lge.) Sing., *Armillariella mellea* (Vahl. in Fl. Dan. ex Fr.) Karst., *Bjerkandera fumosa* (Pers. ex Fr.) Karst., *Bjerkandera adusta* (Willd. ex Fr.) Karst., *Botrytis cinerea* Pers. ex Fr., *Cerrena unicolor* (Bull. ex Fr.) Murr., *Crucibulum laeve* (Bull. ex Fr.) Kambly, *Flammulina velutipes* (Curt. ex Fr.) Sing., *Fomes fomentarius* (L. ex Fr.) Kickx., *Gleophyllum odoratum* (Wulf. ex Fr.) Imaz., *Heterobasidion annosum* (Fr.) Bref., *Inonotus obliguus* (Pers. ex Fr.) Pil., *Inonotus radiatus* (Sow. ex Fr.) Karst., *Kuehneromycetes mutabilis* (Schaeff. ex Fr.) Murill., Laetiporus sulphureus Bull. ex Fr., *Lentinus edodes* (Berk.) Sing., *Lentinus lepideus* Fr., *Lepista nuda* (Bull. ex Fr.) Cooke, *Microascus senegalensis* Arx., *Mycena aurantiomarginata* (Fr.) Lev., *Ophiostoma acoma* Nannf., *Panus tigrinus* (Bull. ex Fr.) Sing., *Pestaliota* sp., *Phaeolepiota aurea* (Matt. ex Fr.) Mre., *Phanerochaete chrysosporium* Burdsall, *Phellinus igniarius* (L.) Quel., *Phellinus pini* (Bratere ex Fr.) Ames, *Phlebia radiata* Fr., *Pholiota glutinosa* Kawamura, *Pleurotus eryngii* (Jacq. ex Fr.) Quel., *Pleurotus florida* (Fr.) Kumm., *Pleurotus ostreatus* (Jacq. ex Fr.) Kumm., *Pleurotus pulmonarius* (Fr.) Quel., *Pleurotus sajor-caju*, *Poria albidofusca* Doman., *Poria crustulina* Bres., *Schizophyllum communae* Fr., *Serpula lacrymans* (Wulf. ex Fr.) Schreet, *Spherobolus stellatus* Tode ex Pers., *Spongiporus guttulatus* (Schrad. ex Fr.) Karst., *Sporongis fragilis* (Fr.) Karst., *Sporotrichum pulverulentum* Novobranova, *Stereum chailetti* (Pers.) Fr., *Stereum chirsutum* (Willd. ex Fr.) Pers, *Trametes versicolor* (L. ex Fr.) Pil., and *Trichaptum abietinum* (Pers. ex Fr.) Ryv.. Culture medium was Czapek Dox and Lindeberg media containing differ-

ent amounts of Na_2HPO_4 (Bollag & Leonowicz, 1984). The media pH was adjusted by phosphate to the optimal values for particular fungal cultures. The culture solutions were grown in static conical flasks at 27℃ till the mycelium occupied the whole surface of the liquid.

The mycelial mats were collected and homogenized in a Waring Blender. The shallow stationary cultures, after inoculation with 4%(v/v) of the homogenate, were incubated in 250*ml* conical flasks containing 50*ml* medium or (for the enzyme preparation) in 1000*ml* flat (Roux type) flasks with 200*ml* medium at 27℃. The inducers (aromatic compounds) were added when the mycelium covered about 1/4 volume of the culture surface in a final concentration of 0.2mM. The compounds sparsely soluble in water were dissolved in a small amount of 0.1M NaOH. The pH of the solutions was adjusted to the initial pH 5.5 with 0.1M HCl and the solutions were sterilized by filtration through Sterivex-GS 0.2mm filters (Millipore, Bedford, Massachusetts).

Laccase activity was measured at 20℃ on a Shimadzu Graphic Printer PR-1 spectrophotometer with syringaldazine as a substrate, but MES-NaOH buffer utilized by Leonowicz and Grzywnowicz(1981) was replaced by 0.1M citrate-phosphate buffer(Bollag & Leonowicz, 1984). To exclude endogenous peroxide, the 10min. preincubation(stirring) of the enzyme sample with catalase (10mg/ml) was performed. The activity was expressed in the international units, i.e. in nkat/L, by assuming a molar absorption coefficient of 65,000 (Leonowicz & Grzywnowicz, 1981) for the colored (red) reaction product. It was calculated as follows ;

$$\text{Act} = \frac{dA_{525} \times \text{Total volume(ml)} \times 10^9}{E(\text{syring.}) \times dt \times \text{Sample volume(ml)}} \quad \frac{\text{nkat}}{\text{l}}$$

where; $\text{kat} = \frac{\text{mol}}{\text{S}}$

E(syringaldazine) : 65,000,

S = dt = seconds (= 60).

When the total volume = 1*ml* and the sample vol = 0.1*ml*, then:

$$\text{Act} = dA_{525} \times 2564 \ \frac{\text{nkat}}{\text{l}}$$

3. RESULTS & DISCUSSION

Laccase is known to occur in some fungi as constitutive and inducible forms. An excess of saccharose or glucose in the liquid medium eliminated the spontaneous induction of laccase. These media allow the constitutive production of laccase by fungi, whereas the biosynthesis of the induced enzyme form is repressed by sugar(Bollag & Leonowicz, 1984; Manzanares *et al.*, 1995).

Table 1. Screening of *Trametes versicolor* strains for laccase production.*

Trametes versicolor (Strain No.)	Growth period (days)	Laccase activity (nkat/ *l*)
7	39	654
9	35	478
20	36	927
38	38	564
138	32	647
200	37	815
230	39	687

Notes; * The fungi were grown shallow stationary in the sugar rich medium at 27℃ and the laccase activity was assayed every day to determine the period of growth at the optimum enzyme activity.

At the beginning of our experiments, several aromatic compounds were investigated on their ability to induce the laccase of *T. versicolor* which was our reference fungus. This fungus is well known both as a strong laccase producer (Bourbonnais *et al.*, 1995) and its ability to degrade lignin. As we have 7 different passages of this strain in our collections, we performed their screenings for laccase production. The results shown in Table 1 point to *T. versicolor* strain No. 20 as the best laccase producer. This is the reason why this strain was used as the reference in our further examinations of several aromatic compounds for their inducing ability.

According to the results presented in Table 2, three

Table 2. Effect of aromatic compounds (inducers) on the laccase production by shallow stationary culture of *T. versicolor*.

Inducer	Laccase activity (nkat/ *l*)	Effect of induction on laccase activity (%)
Chlorogenic acid	9672	1118.1
Coniferyl alcohol	2138	169.3
Gallic acid	7698	869.5
Guaiacol	1245	56.8
Caffeic acid	12952	1531.2
Ferulic acid	29875	3662.6
Orcinol	995	25.3
Pentachlorophenol	24563	2993.6
Protocatechuic acid	11689	1372.2
Sinapic acid	19987	2417.3
Syringic acid	18743	2260.6
Syringyl aldehyde	12674	1496.2
Vanillic acid	9453	1090.6
Vanillin	5642	610.6
Veratraldehyde	3549	347.0
Veratric acid	4672	488.4
2,5-xylidine	30298	3915.9
Control (without inducer)	794	100.0

compounds, ferulic acid, pentachlorophenol and 2,5-xylidine, showed very high inducibility of laccase. Other compounds induced the enzyme to a smaller extent. Among the compounds tested, higher inducing effect is shown by acids type than by their corresponding aldehydes. For further experiments, ferulic acid was chosen as the laccase inducer of assayed fungi. Indeed this compound, being an oxidative product of lignin degradation, appears in the environment of fungal attack on wood. Other compounds are much more toxic than ferulic acid and therefore dangerous both for fungi and human health. Ferulic acid was used earlier as a laccase inducer in our laboratory for the three fungi: *P. mutabilis*, *P. ostreatus* and *T. versicolor*(Leonowicz & Trojanowski, 1975a; Leonowicz & Trojanowski, 1975b; Leonowicz *et al.*, 1978).

For screening of fungi for laccase production and its inducibility, the enzymatic activity of the medium was studied during growth of the fungi in non-induced and ferulic acid-induced cultures. The effect of ferulic acid on laccase activity can be seen in Fig. 1.

The fungal strains tested can be divided into five groups. Into the first group we included *B. fumosa* (Pers. ex Fr.) Karst., *I. obliguus* (Pers. ex Fr.) Pil., *L. nuda* (Bull. ex Fr.) Cooke, *L. lepideus* Fr., *P. tigrinus* (Bull. ex Fr.) Sing., *P. aurea* (Matt. ex Fr.) Mre., *P. chrysosporium* Burdsall, *P. eryngii* (Jacq. ex Fr.) Quel., *S. lacrymans* (Wulf. ex Fr.) Schreet, and *S. stellatus* Tode ex Pers.. All of fungi, listed in this first group, although belonging to the wood rotting pathogens, does not show any laccase activity. Most of them (with the exception for *L. lepideus*, *P. tigrinus* and *P. chrysosporium*) probably have never been reported as lignin destroyers. *P. chrysosporium* have been known as an organism lacking in laccase activity, but recently was reported to be a producer of a little amount of extracellular enzyme (Srinivasan *et al.*, 1995). Such achievement was possible in a low nutrient medium containing cellulose as a carbon and ammonium tartrate as a nitrogen source. Our sugar-rich media excluded such a possibility.

The second group of fungi, which contains only three strains, produces laccase, but ferulic acid, instead of induction, repressed the enzyme activity. Such an effect was also observed earlier, in the case of ascomycete fungi, e.g. *Podospora anserina* (Bollag & Leonowicz, 1984). This group of fungi probably does not destroy lignin at all.

The third, biggest group of fungi, also shows laccase activity, but it is neither repressed nor induced by ferulic acid. Some of these fungi (*B. adusta*, *L. sulphureus*, *L. edodes*) are known to be active in lignin transformation (Eriksson *et al.*, 1990; Buswell *et al.*, 1995).

The fourth group of fungi shows both relatively low laccase activity and low induction of the enzyme by ferulic acid. In this group, similarly to the former one, some of the fungi (*I. radiatus*, *P. albidofusca*, *S. commune*, and *S. chailetti*) are known to be active in lignin transformation.

The fifth group represents the fungi possessing a very high laccase activity, and the enzyme is usually strongly induced by ferulic acid. Among the strains of this group there are found such heavy destroyers of lignin like *T. versicolor* (Bournnais *et al.*, 1995), *P.*

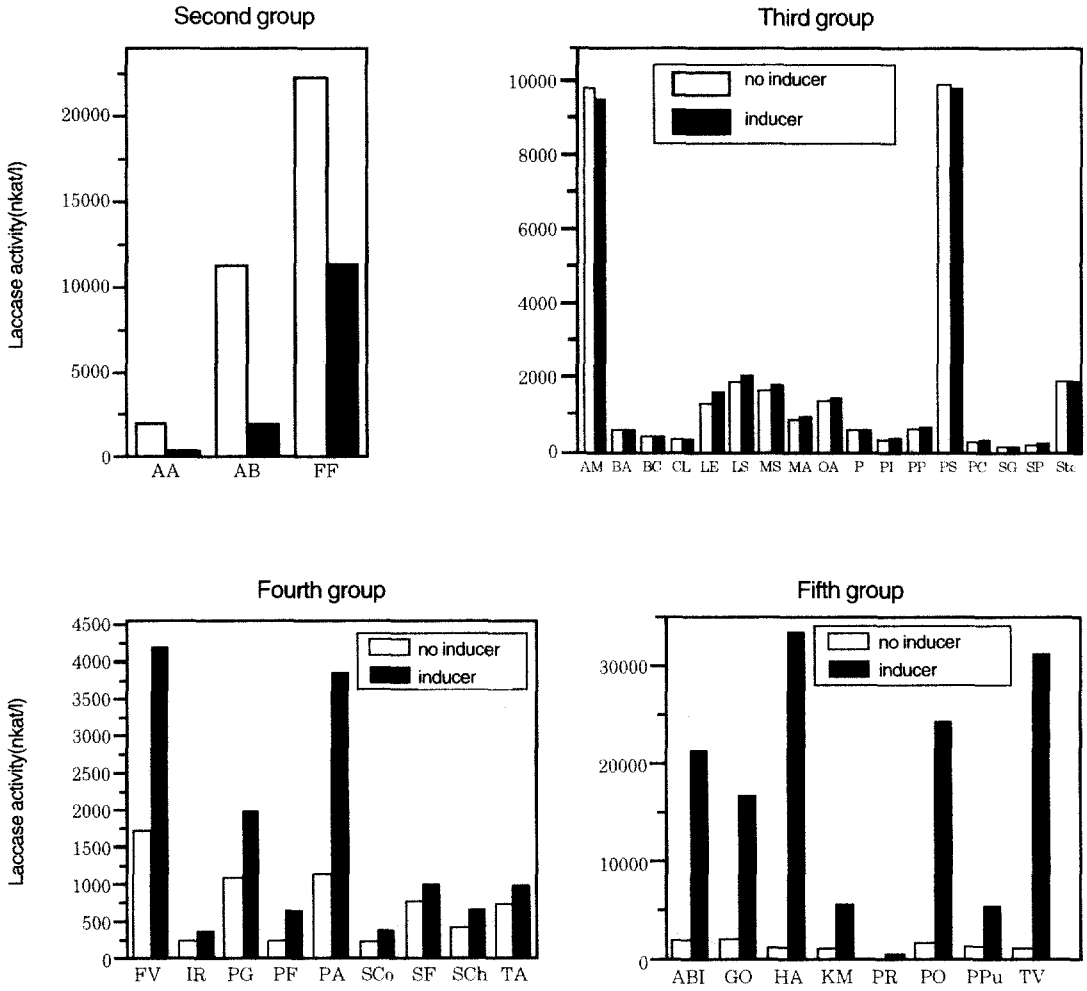

Fig. 1. Effect of ferulic acid addition on laccase inducibilities by the shallow stationary cultures of screened fungi.

Notes; AA : *Agaricus arvensis*, AB : *Agaricus bisporus*, FF : *Fomes fomentarius*, AM : *Armillariella mellea*, BA : *Bjerkandera adusta*, BC : *Botrytis cinerea*, CL : *Crucibulum laeve*, LE : *Lentinus edodes*, LS : *Laetiporus sulphureus*, MS : *Microascus senegalensis*, MA : *Mycena aurantiomarginata*, OA : *Ophiostoma acoma*, P : *Pestaliota sp.*, PI : *Phellinus igniarius*, PP : *Phellinus pini*, PS : *Pleurotus sajor-caju*, PC : *Poria crustulina*, SG : *Spongiporus guttulatus*, SP : *Sporotrichum pulverulentum*, StC : *Stereum chirsutum*, FV : *Flammulina velutipes*, IR : *Inonotus radiatus*, PG : *Pholiota glutinosa*, PF : *Pleurotus florida*, PA : *Poria albidofusca*, SCo : *Schizophyllum communae*, SF : *Sporongis fragilis*, SCh : *Stereum Chailetti*, TA : *Trichaptum abietinum*, ABi : *Abortiporus biennis*, GO : *Gleophyllum odoratum*, HA : *Heterobasidion annosum*, KM : *Kuehneromycetes mutabilis*, PR : *Phlebia radiata*, PO : *Pleurotus ostreatus*, PPu : *Pleurotus pulmonarius*, TV : *Trametes versicolor*.

radiata, *K. mutabilis*, *P. ostreatus* and *H. annosum*. In the fifth group of fungi there appears also *C. unicolor* showing lower laccase inducibility than other fungi. This fungus, however, possesses very high constitutive laccase, the highest of all the fungi tested. Its level is comparable to that in the *T. versicolor* induced

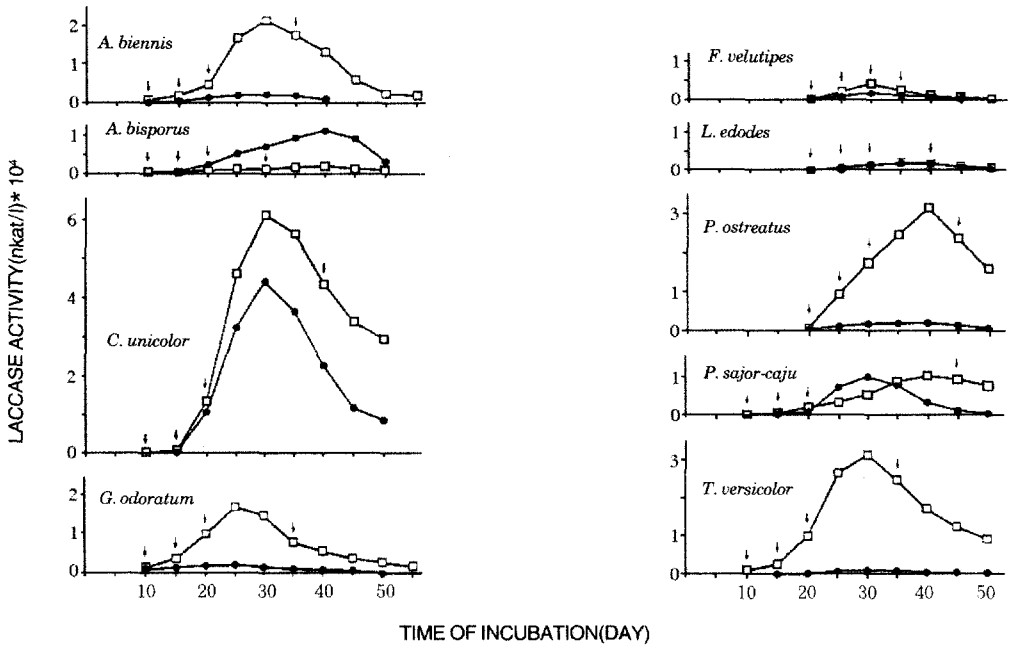

Fig. 2. Examples of extracellular laccase production during the growth of fungi in non-induced (●) and ferulic acid induced (□) cultures. The arrows show when ferulic acid was added to the growth medium.

culture. This fungus is known as a producer of a very large amount of inducible laccase (Agematu *et al.*, 1993; Crawford, 1981; Bollag & Leonowicz, 1984).

It was shown that also others than *C. unicolor* fungi produce a very large amount of laccase. About ten or more than ten thousand nkat/liter of active laccase are produced by *A. biennis* (in induced culture), *A. bisporus* (non-induced), *A. mellea* (both induced and non-induced cultures), *F. fomentarius* (both), *G. odoratum* (induced), *P. ostreatus* (induced), *P. sajor-caju* (both) and *T. versicolor* (induced culture). Two of these laccase rich fungi, namely *A. biennis* and *G. odoratum* probably have never been reported as possessing laccase. So far fewer than 30 species have been investigated on this enzyme(Tuor *et al.*, 1995) including its inducibility(Vasdev & Kuhad, 1994).

The examples of production of the constitutive and inducible enzymes by tested fungi are shown in Fig. 2. After addition of ferulic acid when there is a declining trend of the enzyme activity no further induction of laccase is seen. This phenomenon is observed in almost all of the fungi tested. It may originate from the specific "saturation of laccase capacity". Each strain of fungi possesses probably the "genetically limited capacity" of the production of particular enzyme proteins. Otherwise, non controlled over-production of one enzyme could kill the fungal cell. Ferulic acid is not so great an inducer of laccase production in *C. unicolor*, as it is in *P. ostreatus*, or *T. versicolor*. However, it can be clearly seen that the best source of laccase is *C. unicolor*. Its activities both in the non-induced and induced cultures exceed 40 and 60 thousand nkat/liter, respectively.

4. CONCLUSIONS

Screening of wood rotting fungi for extracellular laccase production and its inducibility by various inducers were made. Among tested 17 compounds, ferulic acid, pentachlorophenol and 2,5-xylidine showed very high inducibility of laccase. Other compounds, such as sinapic acid, syringic acid and coffe-

ic acid, also have the laccase induced relatively high. Among these compounds, higher inducing effect is shown by acids than by their corresponding aldehydes. For further experiments, ferulic acid was chosen as the laccase inducer of assayed fungi, because this compound, being an oxidative product of lignin degradation, appears in the environment of fungal attack on wood.

Thirty eight strains among 48 fungal species tested, produced laccase. The enzyme in 32 cases can be induced by ferulic acid (in 11 cases considerably). This result extends the list of known laccase producing fungi and their inducibility. From this point of view new species including *Abortiporus biennis* and *Gleophyllum odoratum*, where highly induced laccase by ferulic acid, were found.

The best source of the constitutive laccase is *Cerrena unicolor*. Its constitutive enzyme activity is the highest among all fungi tested and is comparable to inducible one in *Abortiporus biennis*, *Gleophyllum odoratum* or *Trametes versicolor*. However, to reach such high laccase activity *Cerrena unicolor* does not need any toxic inducer for the environment.

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