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The Selective Visualization of Lignin Peroxidase, Manganese Peroxidase and Laccase, Produced by White Rot Fungi on Solid Media

Won Youl Ryu, Moon Yup Jang, and Moo Hwan Cho*

School of Chemical Engineering & Technology, Yeungnam University, Gyongsan, Gyongbuk 712-749, Korea

Abstract A visual method for the selective screening of lignin degrading enzymes, produced by white rot fungi (WRF), was investigated by the addition of coloring additives to solid media. Of the additives used in the enzyme production media, guaiacol and RBBR could be used for the detection of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. Syringaldazine and Acid Red 264 were able for the detection of both the MnP and laccase, and the LiP and laccase, respectively, and a combination of these two additives was able to detect each of the ligninases produced by the WRF on solid media.

Keywords: lignin peroxidase, manganese peroxidase, laccase, color additives, white rot fungi

INTRODUCTION

White rot fungi (WRF) have been widely studied in order to obtain ligninolytic enzymes useful for biotechnological applications. Two peroxidase groups, lignin peroxidase (LiP) and manganese peroxidase (MnP), and laccase, are known to be associated with ligninolytic activity [1]. Ligninolytic cultures of WRF secreted LiP, MnP and laccase, in various concentrations [2]. These enzymes have the ability to mineralize lignin, and various other xenobiotic compounds, such as polycyclic hydrocarbons (PAHs), chlorinated phenols, polychlorinated biphenyls (PCBs), dioxin, alkylhalides, nitrotoluenes and dyes [3-10]. However, in the studies on the production of ligninolytic enzymes, only a few species of WRF have been used. Therefore, many researchers have stressed the need to investigate the ligninolytic enzyme systems of other WRF. To this end, several screening methods, involving dyes, such as Poly R-411, Poly R-418, and Remazol Brilliant Blue R (RBBR), have been reported [11]. Pasti-Grigsby *et al.* observed a correlation between the decolorization of RBBR and the ligninonytic ability of microorganisms [12]. Glenn and Gold demonstrated that the decolorization of polymeric dyes in liquid culture was related to the lignin degradation system [11]. The evidence presented indicated that these dyes serve as substrates for the fungal lignin degradation system. The use of dyes in a solid medium has been proved as a method for screening the ligninolytic activity of a large number of microorganisms, and their mutants or clones [13]. Pasti-Grigsby *et al.* also reported that anthrone-type dyes were suitable substrates for the analyses of peroxidases produced in liquid cultures by *Streptomycetes* [12]. It has also been reported that the decolorization of polymeric dyes, by *Phanerochaete chrysosporium*, indicates the presence of LiP in solid media [11]. However, a simple method for selectively screening one of the lignin degrading enzymes, produced by WRF in solid media, has not been reported. Therefore, this study was undertaken to prove that certain additives to solid media could be used to this end in various ligninases production media.

***Corresponding author**
Tel: +82-53-810-2517 Fax: +82-53-814-8790
e-mail: mhcho@yumail.ac.kr

MATERIALS AND METHODS

Chemicals

The 4-hydroxy-3,5-dimethyoxy-benaldehydeazne (syringaldazine), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2-methoxy-phenol (guaiacol), Remazol Brilliant Blue R (RBBR) and Acid Red 264 dyes were all purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). All the other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The LiP, MnP and laccase used through out the experiments were produced from rotary-shaken liquid cultures of *Phanerochaete chrysosporium* and *Trametes* sp.

Strains

Phanerochaete chrysosporium PSBL-1 and *Trametes* sp. (KFCC 10941) were chosen as ligninolytic microorganisms. The *P. chrysosporium* was maintained on 1% (w/v) malt extract agar plates, kept at 37°C, whereas the *Trametes* sp. was maintained at 28°C. All the fungi were kept at 4°C until used.

Table 1. Color changes of the additives due to *P. chrysosporium* on solid media

Additives	Color change*		
	LiP production medium	MnP production medium	Non-ligninase producing medium
RBBR (50 μM)	Decolorization started after 3 days; completed in 7 days	Decolorization started after 4 days; completed in 8 days	No decolorization up to 25 days
Guaiacol (1 mM)	Color in the center of Petri plate changed to dark brown within 4-5 days	Color in the center of Petri plate changed to dark brown within 4-5 days	No decolorization up to 25 days
Syringaldazine (0.1 mM)	No change in color up to 25 days	Color in the center of Petri plate changed to dark brown within 5 days	No decolorization up to 25 days
Acid Red 264 (80 μM)	Decolorization started after 2 days; complete in 4 days	No decolorization up to 25 days	No decolorization up to 25 days

* by visual examination

Solid Culture Media

All the fungi were grown on solid media, containing different additives (Acid Red 264 80 μM, guaiacol 1 mM, RBBR 50 μM and syringaldazine 0.1 mM) with 1.5% agar, on 8.5 cm diameter Petri plates. The solid culture media were of the same compositions and kept in the same conditions as the liquid culture media described below.

Liquid Culture Media

The basal medium for the production of the LiP and MnP was the nitrogen-limited medium, as described by Tien and Kirk, with 10 g glucose/L, as the carbon source, and 0.2 g ammonium tartrate, as the nitrogen source, with the exception of the dimethyl succinate, which was replaced by 0.1 M succinate buffer (pH 4.5) [14], and for the case of the production of the MnP, 100 mg $MnSO_4 \cdot H_2O$ was added. The basal medium for the production of the laccase was the nitrogen-sufficient liquid medium, with 20 g glucose/L, as the carbon source, and 4 g ammonium tartrate, as the nitrogen source [15,16]. The medium was buffered at pH 4.5 by 2, 2-dimethyl succinic acid (25.3 mM). The *P. chrysosporium*, immobilized on polyurethane foam, was incubated at 37°C at 200 rpm agitation, whereas the *Trametes* sp. was incubated at 28 °C at 200 rpm agitation. The ligninases produced were purified from the culture broth of *P. chrysosporium* for the LiP and MnP, and of the *Trametes* sp. for the laccase. The purified LiP, MnP and laccase, were used in the color changes tests for all the additives. Culture filtrate, not producing any ligninase by *P. chrysosporium*, was used as the control.

Enzyme Assays

Culture broth samples, from the desired cultures, were assayed for the activity of LiP and MnP by the method described by Tien and Kirk [14]. The laccase activity was determined as described by Pedro and Coll [17].

RESULTS AND DISCUSSION

Color Changes of Additives by *P. chrysosporium* on Solid Media

As shown in Table 1, complete color changes with the guaiacol and RBBR were shown in the media producing the LiP and MnP. The color of the syringaldazine changed to a dark brown in the middle of the medium plate within 4-5 days of incubation on the production of the MnP, whereas, no color change was observed with the syringaldazine in the medium used for the production of the LiP until day 25 of incubation. Complete decolorization of the Acid Red 264 was observed after 4 days of incubation in the production media of the LiP, whereas there was no color change in the production media of MnP after 25 days of incubation. A color change with the additives was not observed on the control medium. Of the additives contained in the enzyme production media, only the guaiacol and RBBR could be used for the detection of both the MnP and LiP. The syringaldazine could also be used for the detection of the MnP, and the Acid Red 264 for that of the LiP.

Color Changes of Additives by *Trametes* sp. on Solid Media

As shown in Table 2, complete color changes with the guaiacol and RBBR were shown in all the tested media. The color of the guaiacol changed to a dark brown in the middle of the plate within 4 days of incubation with all the ligninase production media. Complete decolorization of the RBBR was observed after 7 days of incubation in the LiP, MnP and laccase production media. The color change with the syringaldazine started after 2 days of

Table 2. Color changes of the additives due to *Trametes* sp. on solid media

Additives	Color change*		
	LiP production medium	MnP production medium	Laccase production medium
RBBR (50 μM)	Decolorization started after 3 days; completed in 7 days	Decolorization started after 4 days; completed in 7 days	Decolorization started after 4 days; completed in 7 days
Guaiacol (1 mM)	Color in the center of Petri plate changed to dark brown within 3 days	Color in the center of Petri plate changed to dark brown within 4 days	Color in the center of Petri plate changed to dark brown within 4 days
Syringaldazine (0.1 mM)	No change in color up to 25 days	Color changed to dark brown within 4 days	Color changed to dark brown within 4 days
Acid Red 264 (80 μM)	Decolorization started after 3 days; completed in 6 days	No decolorization up to 25 days	Decolorization started after 3 days; completed in 6 days

* by visual examination

Table 3. Color changes of the additives due to ligninases in liquid media

Enzyme/Additives	Activities of enzymes (U/L)	Color change*
LiP		
RBBR (50 μM)	120	Complete decolorization in 5 days
Guaiacol (1 mM)	50	Color changed to dark brown within 4 days
Syringaldazine (0.1 mM)	100	No change in color
Acid Red 264 (80 μM)	150	Complete decolorization in 4 days
MnP		
RBBR (50 μM)	150	Complete decolorization in 5 days
Guaiacol (1 mM)	60	Color changed to dark brown within 5 days
Syringaldazine (0.1 M)	110	Color changed to dark brown within 5 days
Acid Red 264 (80 μM)	200	No change in color
Laccase		
RBBR (50 μM)	80	Complete decolorization in 7 days
Guaiacol (1 mM)	50	Color changed to dark brown in 4 days
Syringaldazine (0.1 mM)	50	Color changed to dark brown in 4 days
Acid Red 264 (80 μM)	160	Complete decolorization in 5 days

* by visual examination

incubation with the MnP and laccase production medium. The color of the syringaldazine changed, at first, to a dark brown in the middle of the plate, within 4 days of incubation for the production media of MnP and laccase, and then decolorized after 7-8 days of incubation, whereas no color change on the LiP production medium was observed. Decolorization of the Acid Red 264 was shown within 3 days of incubation in the LiP and laccase production media, whereas no decolorization of the Acid Red 264 was found in the production medium of the MnP until 23 days of incubation. Of the additives contained in the enzyme production media, the guaiacol and RBBR could be used for the detection of all the ligninases, and the syringaldazine and Acid Red 264 could be used for the detection of the laccase. The syringaldazine could also be used for the detection of both the MnP and laccase, and the Acid Red 264 for both the LiP and laccase.

Color Changes of Additives by WRF in Liquid Media

The activities of the LiP, MnP and laccase were determined in parallel, with the color changes of additives in the liquid media, over the time of the batch cultures. As shown in Table 3, the RBBR was completely decolorized within 7 days of incubation with an LiP concentration of 120 U/L, a MnP concentration of 150 U/L or laccase concentrations of 80 U/L and 180 U/L, whereas there was no decolorization of the RBBR after 10 days of incubation under non-ligninase producing conditions. The color of guaiacol changed to a dark brown within 4-5 days of incubation with a LiP concentration of 50 U/L, a MnP concentration of 60 U/L or laccase concentrations of 100 U/L and

Table 4. Summary for the color changes of the additives by each ligninase

Additives	Color change		
	LiP	MnP	Laccase
RBBR	O	O	O
Guaiacol	O	O	O
Syringaldazine	×	O	O
Acid Red 264	O	×	O

(O : change, × : no change)

150 U/L, whereas the color of guaiacol did not change after 10 days of incubation in the absence of ligninases. The colors of the guaiacol and RBBR changed in the LiP, MnP and laccase production media, which confirmed the previously reported results of other researchers [18-21]. The color of the syringaldazine remained unchanged after 10 days of incubation with a LiP concentration of 100 U/L, whereas the syringaldazine changed to a dark brown, within 5 days of incubation, with a MnP concentration of 110 U/L, or a laccase concentration of 1,000 U/L. It has been reported previously that the syringaldazine was a specific substrate for both the MnP and laccase [22]. The color of the Acid Red 264 remained unchanged after 10 days of incubation with a MnP concentration of 250 U/L, whereas it was decolorized within 4-5 days of incubation with a laccase concentration of 200 U/L and a LiP concentration of 150 U/L. As described above, the results of color changes in the liquid cultures were the same as those of the agar plate cultures.

Color Change of Additives by Purified LiP, MnP, and Laccase

When the purified laccase (1.0 U/mL) was used for in vitro tests, the colors of all the four additives changed completely with in 1-2 min. Conversely, when MnP (1.0 U/mL) was used for in vitro tests, no decolorization of the Acid Red 264 was observed, even with a dose as high as 2.0 U/mL. However, the colors of RBBR, guaiacol and syringaldazine were all completely changed by the MnP within 1-2 min. When LiP (1.0 U/mL) was used for in vitro tests, the colors of the RBBR, guaiacol and Acid Red 264 were completely changed within 1-2 min, whereas the color of syringaldazine remained unchanged. Therefore, the results of color changes due to the purified LiP, MnP and laccase were the same as those of the solid and liquid cultures, as summarized in Table 4.

CONCLUSION

As the colors of RBBR and guaiacol are changed by all the ligninases, they are not able to be used for the separate detection of the ligninases, but can be used for screening WRF that produce ligninase. Conversely, the syringaldazine and Acid Red 264 can be combined to screen for each ligninase produced by WRF separately. If a strain of WRF, cultured on two solid media containing the syringaldazine and Acid Red 264, respectively, changes the colors of both the additives, it is known that it produces the laccase only. If a strain of WRF does not change the color of the syringaldazine, but change that of the Acid Red 264, it is known that it produces the LiP only. If a strain of WRF does not change the color of the Acid Red 264, but change that of the syringaldazine, it is known that it produces the MnP only. In conclusion, a simple and selective visual method was developed for the detection of each ligninase produced by WRF on solid media, by the combined use of the syringaldazine and Acid Red 264 as color additives.

REFERENCES

[1] Tien, M. and T. K. Kirk (1983) Lignin-degrading enzyme from hymenimycete *Phanerochaete chrysosporium*. *Science* 221: 661-663.

[2] Hatakka, A. (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *FEMS Microbiol.* 13: 125-135.

[3] Baker, W. L., K. Sabapathy, M. Vibat, and G. Lonergan (1996) Laccase catalyzes formation of an indamine dye between 3-methyl-2-benzothiazolinone hydrazone and 3-dimethylaminobenzoic acid. *Enzyme Microb. Technol.* 18: 90-94.

[4] Bezarel, L., Y. Hadar, and C. E. Cerniglia (1996) Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 62: 292-295.

[5] Bollag, J. M., K. L. Shuttleworth, and D. H. Anderson (1988) Laccase-mediated detoxification of phenolic compounds. *Appl. Environ. Microbiol.* 54: 3086-3091.

[6] Bumpus, J. A., M. Tien, D. Wright, and S. Aust (1985) Oxidation of persistent environmental pollutants by a white rot fungus. *Science* 228: 1434-1436.

[7] Bumpus, J. A. and B. Brock (1988) Biodegradation of crystal violet by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Envion. Microbiol.* 58: 3598-3604.

[8] Cerniglia, C. E., P. P. Fu, and S. K. Yang (1982) Metabolism of 7-methyl-benzo[a]anthracene and 7-hydroxymethylbenz[a]anthracene by *Cunninghamella ale-*

gance. Appl. Environ. Microbiol. 44: 682-689.

[9] Collins, P. J., M. J. J. Kotterman, J. A. Field, and A. D. W. Dobson (1996) Oxidation of anthracene and benzo[a]pyrene by laccase from *Trametes versicolor*. *Appl. Environ. Microbiol.* 62: 4563-4567.

[10] Ryu, W. R., Y. S. Seo, Y. K. Chang, and M. H. Cho (2000) Biodegradation of polycyclic aromatic hydrocarbons by white rot fungi. *Korean. J. Biotech. Bioeng.* 15(3): 262-267.

[11] Glenn, J. K. and M. H. Gold (1983) Decolorization of several polymeric dyes by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 45: 1741-1747.

[12]Pasti-Grigsby, M. B., A. Paszczynski, S. Goszczynski, D. L. Crawford, and R. L. Crawford (1992) Influence of aromatic substitution pattern on azo dye degradability by *Streptomyces* spp. and *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 58: 3605-3613.

[13] Chahal, D. S., D. Kluepfel, R. Morosoli, F. Shareck, S. Laplante, and D. Rouleau (1995) Use of dyes in solid medium for screening ligninolytic activity of selective actinomycetes. *Appl. Biochem. Biotechnol.* 51: 137-144.

[14] Tien M and T. K. Kirk (1988) Lignin peroxidase of *Phanerochaete chrysosporium*. *Methods Enzymol.* 161: 238-249.

[15] Ryu, W. R., S. H. Shim, M. Y. Jang, Y. J. Jeon, K. K. Oh, and M. H. Cho (2000) Biodegradation of pentachlorophenol by white rot fungi under ligninolytic and nonligninolytic conditions. *Biotechnol. Bioprocess Eng.* 5: 211-214.

[16] Seo, Y. S., W. R. Ryu, Y. K. Chang, and M. H. Cho (2000) Biodegradation of polycyclic aromatic hydrocarbons using immobilized cells of *Phanerochaete chrysosporium*. *Korean. J. Biotechnol. Bioeng.* 15: 247-253.

[17] Pedro, M. and J. M. Coll (1993) Purification and characterization of phenoloxidase (Laccase) from the lignin-degrading basidiomycete PM1. *Appl. Environ. Microbiol.* 59: 2607-2613.

[18] Addleman, K., T. Dumonceaux, M. G. Paice, R. Bourbonnais, and F. S. Archibald(1995) Production and characterization of *Trametes versicolor* mutants unable to bleach hardwood kraft pulp. *Appl. Environ. Microbiol.* 61: 3687-3694.

[19] Ollikka. P., K. Alhonmaki, V. M. Lepparen, T. Glumoff, T. Raijola, and I. Suominen (1993) Decolorization of azo, triphenyl methane, heterocyclic, and polymeric dyes by lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 59: 4010-4016.

[20] Schliephake, K. and G. T. Lonegran (1996) Laccase variation during dye decolorization in a 200 L packed-bed bioreactor. *Biotechnol. Lett.* 18: 881-886.

[21] Vyas, B. R. M. and H. P. Molitoris (1995) Involvement of extracellular H_2O_2-dependent ligninolytic activity of the white rot fungus *Pleurotus ostreatus* in the decolorization of Remazol Brilliant Blue R. *Appl. Environ. Microbiol.* 61: 3919-3927.

[22] Thurston, C. F. (1994) The structure and function of fungal laccases. *Microbiology* 140: 19-26.

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