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Characterization of Dye Decolorization in Cell-Free Culture Broth of *Trametes versicolor* CBR43

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The dye decolorization rate in a cell-free culture broth of the white-rot fungus *Trametes versicolor* CBR43 was studied, including the effects of inhibitors of NaCl, Zn(II), and Cd(II) on dye decolorization activity. The maximum rates of dye decolorization in cell-free culture broth were 1,410, 44.7, 41.2, and 0.19 $\mu mol \cdot l^{-1} \cdot min^{-1}$ for Acid Blue 62, Acid Black 175, Reactive Blue 4, and Acid Red 114, respectively. The inhibition effects of NaCl, Zn(II), and Cd(II) on dye decolorization were quantitatively compared using the half maximal inhibition concentration (IC_{50}), which indicates the concentration of an inhibitor required for 50% inhibition. Based on IC_{50} values, dye decolorization in the cell-free culture broth of CBR43 was most potently inhibited by Cd(II), whereas the inhibitory effect of NaCl was relatively low. The dye decolorization rates and IC_{50} data can be used in the design and development of a dye-wastewater treatment process using *T. versicolor* CBR43 and its operating factors.

Keywords: *Trametes versicolor*, cell-free culture broth, decolorization, kinetic analysis, inhibitor

White-rot basidiomycetes are filamentous fungi that decompose the cellulose, hemicellulose, and lignin of wood. White-rot basidiomycetes decompose lignin via ligninolytic enzymes (laccase, Mn-dependent peroxidase, lignin peroxidase, dye-decolorizing peroxidase, *etc*.) and coenzymes (H_2O_2-generating enzyme, glyoxal oxidase, *etc*.). Because ligninolytic enzymes have low substrate specificity, mycoremediation, which degrades xenobiotics such as dyes and polycyclic aromatic compounds using those enzymes, is receiving attention [1–3].

Among white-rot basidiomycetes, the genus *Trametes* belonging to the family Polyporaceae and the class Agaricomycetes uses deciduous trees, such as oak, as a host and commonly grows in tiled layers [4]. The cap is rust-brown or darker brown, sometimes with blackish zones. The genus *Trametes* is known to be able to decolorize dyes used in textile dyeing [4]. *T. versicolor* decolorized Acid Black and Acid Violet 7 by 97% and 11%, respectively, in 9 days [5]. In addition, this fungus decolorized Reactive Black 5 by 16% in 9 days [6]. *Trametes* sp. isolated from temperate forests also decolorized azo, anthraquinone, and triphenylmethane dyes in the range of 37–100% in 5 days [7].

The biggest advantage of using white-rot basidiomycetes-containing *Trametes* sp. for decolorization of dye wastewater is that very cheap agricultural and forestry waste, such as waste wood, rice straw, and rice bran, can be used as cultivation feedstocks [4]. The main components of agricultural and forestry waste are non-biodegradable polymers (cellulose, hemicellulose, and lignin), and white-rot basidiomycetes can decompose those polymers using extracellular enzymes and then use the degradation products for growth. Dye wastewater can be effectively decolorized by a culture broth containing the extracellular ligninases produced in the process [1–4].

A solid-state fermentation (SSF) method is most commonly used for the cultivation of basidiomycetes using agricultural and forestry waste as feedstocks [8, 9]. Therefore, for dye wastewater treatment processes using basidiomycetes, it is desirable to construct a 2-stage process: a first stage in which the mycelium and extracellular ligninases and coenzymes are produced in an SSF, and a second stage in which dye wastewater is treated using leachate from the

SSF. Considerable research has been performed on the characterization and evaluation of ligninase production in SSF using sawdust, sorghum husks, wheat bran, wheat straw, rice straw, corn husks, and corn cobs [10–17]. In addition, some researchers have demonstrated dye decolorization using purified laccase, Mn-dependent peroxidase, and lignin peroxidase from the culture broth of white-rot basidiomycetes [2, 4, 7, 8, 18–21]. However, little information is available on dye decolorization using the cell-free culture broth of white-rot basidiomycetes. Therefore, in this study, the kinetics of dye decolorization in the cell-free culture broth of *Trametes versicolor*, a member of the white-rot basidiomycetes showing excellent dye decolorization ability, was investigated. In addition, the inhibitory effects of salt and heavy metals on dye decolorization by *T. versicolor* were evaluated both quantitatively and qualitatively.

In this study, *T. versicolor* CBR43 isolated from Cheonma Mountain, located in Gyeonggi-do, South Korea, was used as an inoculum source [22]. The mycelium of CBR43 was cultivated in PDB medium (potato starch 4 g/l, dextrose 20 g/l) or on PDA medium (15 g/l of agar added to PDB medium). The decolorization tests used three acid dyes (Acid Red 114, Acid Blue 62, and Acid Black 175) and one reactive dye (Reactive Blue 4), all of which are commonly used in the textile dyeing industry. Acid Red 114 and Acid Black 172 are azo-type dyes, and Acid Blue 62 and Reactive Blue 4 are anthraquinone-type dyes.

The cell-free culture broth from CBR43 was prepared as follows. Inoculum was prepared by cutting the mycelium of a CBR43 strain cultured on PDA medium for 5 days into 6-mm-diameter discs using the backside of a sterilized 200 µl pipette tip. Ten inoculums were added to a 250 ml Erlenmeyer flask containing 100 ml of PDB medium. The flask was incubated at 28°C while rotating at 150 rpm for 6 days. The resulting culture broth was centrifuged at 15,000 ×*g* for 10 min at 4°C, and the supernatant was used as the cell-free culture broth.

The effects of dye concentration and inhibitors (NaCl, Zn(II), Cd(II)) on dye decolorization in the cell-free culture broth were also investigated. For this, a 4-fold diluted cell-free culture broth was used for testing the decolorization of Acid Blue 62. For Acid Black 175 and Reactive Blue 4, a 2-fold diluted cell-free culture broth was used. For Acid Red 114, the cell-free culture broth was used without dilution.

To evaluate the dye concentration effect on the decolorization rate, 360 µl of culture broth and 40 µl of each dye solution were placed in a 100-well plate. Dye solution was added to produce a final Acid Blue 4 concentration of 0–300 mg/l, and concentrations of the other three dyes of 0–200 mg/l.

To test the effects of inhibitors on the decolorization reaction, 360 µl of culture broth, 20 µl of dye solution, and 20 µl of inhibitor were added to a 100-well plate. Dye solution was added to a final concentration of 150 mg/l. NaCl, Zn(II), or Cd(II) was added to a final concentration of 0–45, 0–11, or 0–11 mM, respectively. Zn(II) and Cd(II) stock solutions were prepared using $ZnCl_2$ and $CdCl_2$, respectively.

To analyze the decolorization activity, the absorbance was measured at 600 nm for Acid Blue 62, at 580 nm for Acid Black 175 and Reactive Blue 4, and at 495 nm for Acid Red 114 using Bioscreen C (Oy Growth Curves Ab Ltd., Finland). The enzyme reaction temperature was set to 28°C, and the absorbance for Acid Blue 62, Acid Black 175, and

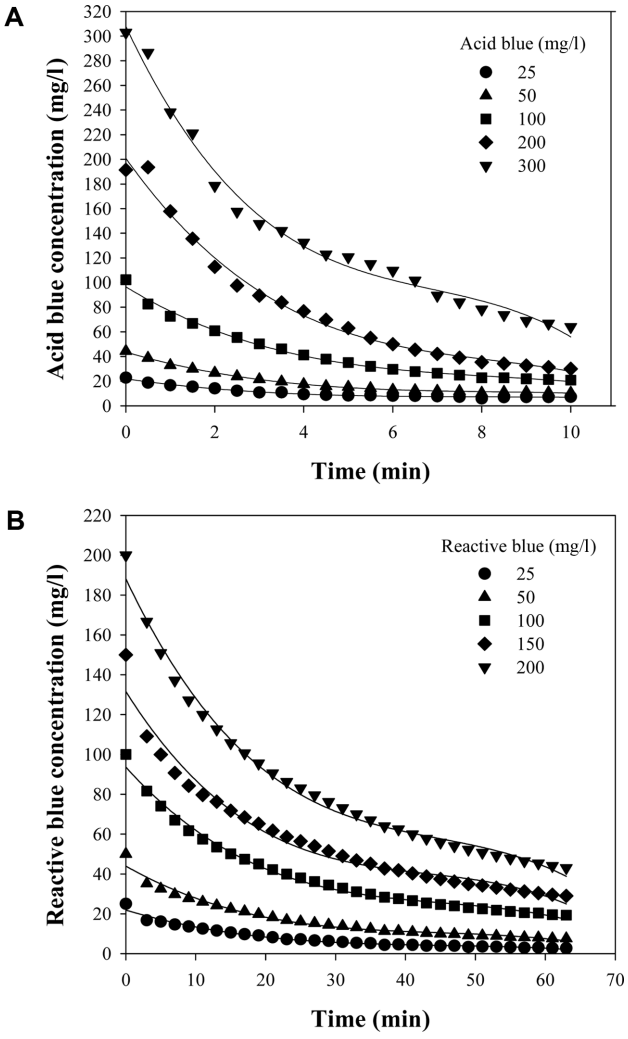

Fig. 1. Time profiles of dye concentration in cell-free culture broth of *T. versicolor* CBR43 at different dye concentrations. (**A**) Acid Blue 62; (**B**) Reactive Blue 4.

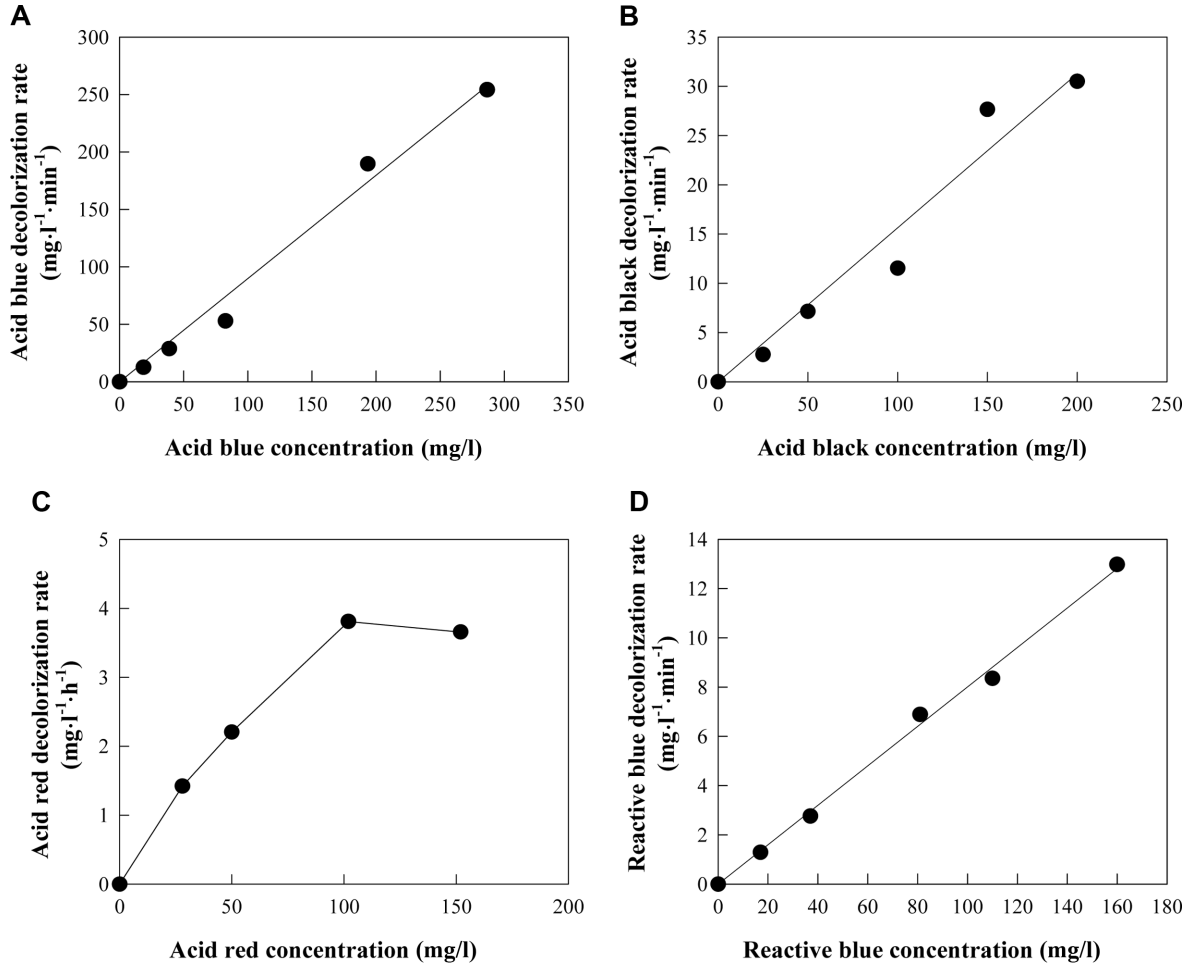

Fig. 2. Effects of dye concentrations on the decolorization rate in cell-free culture broth of *T. versicolor* CBR43.
(**A**) Acid Blue 62; (**B**) Acid Black 172; (**C**) Acid Red 114; (**D**) Reactive Blue 4.

Reactive Blue 4 was measured every 30 sec for 15 min. For Acid Red 114, the absorbance was measured every 20 min for 2 h. Distilled water instead of culture broth was used as a control. All tests were performed in triplicates.

The effects of dye concentration on the decolorization rates of Acid Red 114, Acid Blue 62, Acid Black 175, and Reactive Blue 4 are shown in Figs. 1 and 2. The decolorization patterns of Acid Blue 62 and Reactive Blue 4 in cell-free culture broth are representatively shown in Fig. 1. The dye concentrations decreased with increasing reaction time.

Fig. 2 shows the initial decolorization rates at each dye concentration. Except for Acid Red 114, the decolorization rates of Acid Blue 62, Acid Black 175, and Reactive Blue 4 increased with increasing dye concentration. At a dye concentration of 100 mg/l, the decolorization rates of Acid Blue 62, Acid Black 175, Reactive Blue 4, and Acid Red 114 were 52.85, 11.52, 8.36, and 0.06 $mg \cdot l^{-1} \cdot min^{-1}$ (3.81 $mg \cdot l^{-1} \cdot h^{-1}$), respectively. For Acid Red 114, the decolorization rate increased in proportion to the dye concentration less than 100 mg/l; however, at a dye concentration of 150 mg/l, the decolorization rate decreased to 3.65 $mg \cdot l^{-1} \cdot h^{-1}$. This result suggests that the enzyme activity involved in the decolorization reaction is inhibited at concentrations of Acid Red greater than 150 mg/l.

Table 1. Maximum decolorization rate and saturation constant.

Dye	Maximum decolorization rate ($\mu mol \cdot l^{-1} \cdot min^{-1}$)	Saturation constant (mM)
Acid Blue 62	1,410	1.63
Acid Black 175	44.7	0.25
Acid Red 114	0.19	0.20
Reactive Blue 4	41.2	0.49

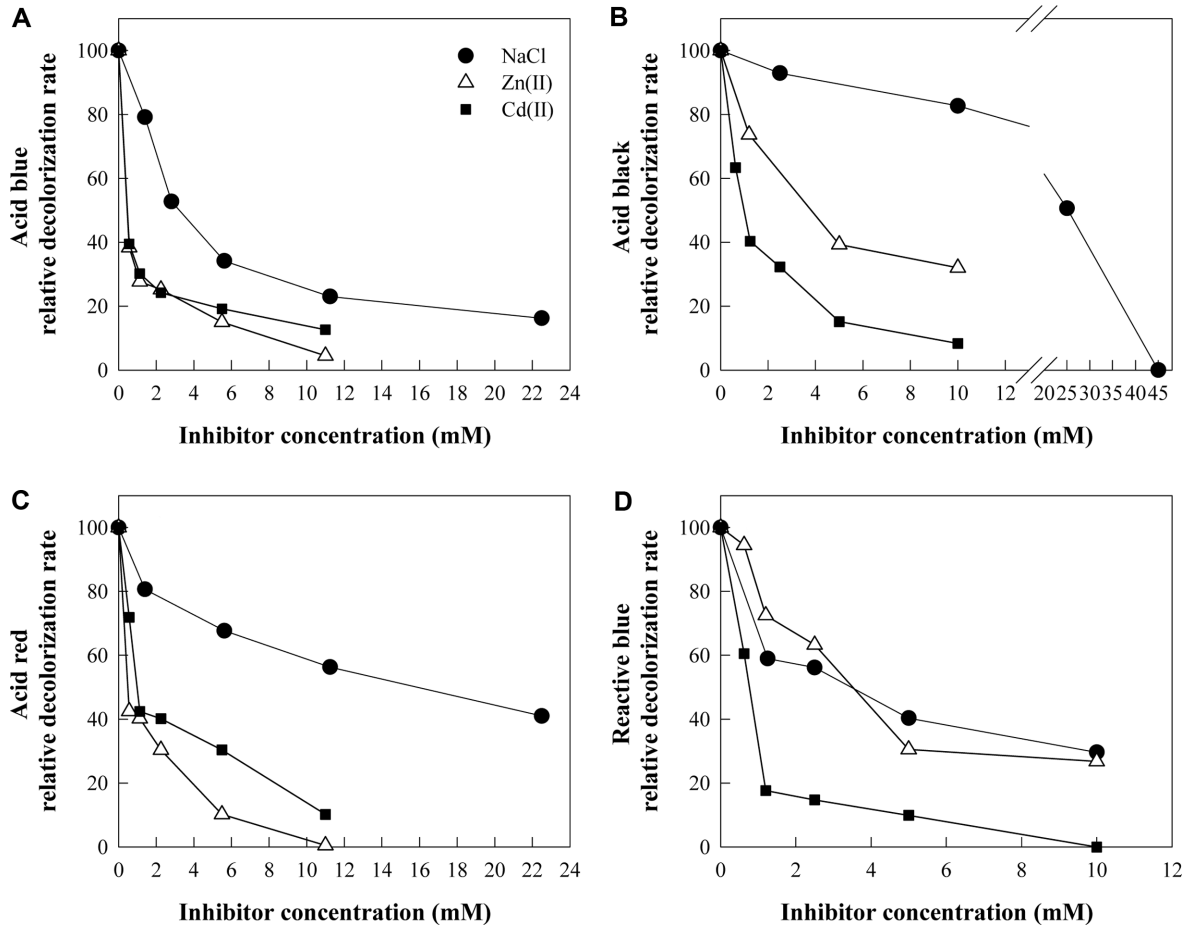

Fig. 3. Effects of NaCl, Zn(II), and Cd(II) concentrations on the decolorization rate in cell-free culture broth of *T. versicolor* CBR43. (**A**) Acid Blue 62; (**B**) Acid Black 172; (**C**) Acid Red 114; (**D**) Reactive Blue 4.

The maximum decolorization rate and the saturation constant for the Michaelis-Menten kinetics were calculated (Eq. (1)) using the decolorization rates at each dye concentration (Table 1).

$$V_0 = \frac{V_{max}[S]}{(K_M + [S])} \quad (1)$$

Here, V_o is the decolorization rate, V_{max} is the maximum decolorization rate, [S] is the dye concentration, and K_M is the saturation constant.

The highest V_{max} of 1,410 μmol·l^{-1}·min^{-1} was found with Acid Blue 62; the V_{max} for Acid Black 175 and Reactive Blue 4 were 44.7 and 41.2 μmol·l^{-1}·min^{-1}, respectively. The lowest V_{max} of 0.19 μmol·l^{-1}·min^{-1} was obtained with Acid Red 114.

Trametes sp. was shown to decolorize anthraquinone dyes, such as Acid Blue 62 and Reactive Blue 4, via laccase [7]. Azoreductase and laccase have been associated with the decolorization of azo dyes such as Acid Black 175 and Acid Red 114 [2]. In general, it is known that bacteria decolorize dyes with azoreductase, whereas fungi use laccase for decolorization [2]. Black Dycem, an azo dye, could be 90% decolorized using laccases from *T. versicolor*, *Ganoderma lucidum*, and *Irpex lacteus* for 48 h [23]. Reactive Black 5, another azo dye, could be decolorized by 43% using the laccase of *T. versicolor* for 30 min [24].

The enzyme involved in decolorization by *Trametes* sp. SQ01 and *T. trogii* Berk S031, which belong to same the genus as the strain used in this study, has been reported to be laccase [7, 25]. In addition, *T. pubescens* has also been shown to decolorize dyes with laccase [20]. In particular, laccase is an important ectoenzyme produced by *T. versicolor* that has decolorized azo, anthraquinone, and indigo dyes [26]. Considering these previous results, it is probable that the dye decolorization in the cell-free culture broth of CBR43 occurs via laccase. However, further study is needed to separate and purify the enzymes involved in dye decolorization in the cell-free culture broth of CBR43.

Because bleaching agents and mordants are used in the

Table 2. Half maximal inhibition concentration (IC_{50}) of NaCl, Zn(II), and Cd(II) on the decolorization rate.

Dye	IC_{50} (mM)		
	NaCl	Zn(II)	Cd(II)
Acid Blue 62	3.11	0.46	0.47
Acid Black 175	22.7	3.97	1.02
Acid Red 114	8.70	0.49	0.98
Reactive Blue 4	3.99	3.12	0.74

fiber dyeing process, dye wastewater contains high amounts of salts and heavy metals [18, 27] that act as inhibitors to the decolorization of dye wastewater. The effects of inhibitors on dye decolorization in the cell-free culture broth of CBR43 are shown in Fig. 3. The decolorization rates for Acid Blue 62 and Reactive Blue 4 were sensitive to inhibition from increasing NaCl concentrations. However, the decolorization rate for Acid Black 175 was unaffected by the addition of 10 mM NaCl, though it was reduced by 50% when adding 20 mM NaCl. The decolorization rates for Acid Blue 62 and Acid Red 114 decreased significantly with an increasing Zn(II) concentration. However, the decolorization rates for Acid Black 62 and Reactive Blue 4 were inhibited relatively little by Zn(II). The decolorization rates for all dyes were remarkably inhibited by Cd(II). Compared with NaCl, Zn(II) and Cd(II) potently inhibited the decolorization ability of the cell-free culture broth.

The half maximal inhibition concentration (IC_{50}), the concentration of an inhibitor required for 50% inhibition, was calculated using the slope in Fig. 3 (Table 2). The IC_{50} values of NaCl for the decolorization rates of Acid Blue 62, Acid Black 175, Acid Red 114, and Reactive Blue 4 were 3.11, 22.70, 8.70, and 3.99 mM, respectively. The IC_{50} values of Zn(II) for the decolorization rates of Acid Blue 62, Acid Black 175, Acid Red 114, and Reactive Blue 4 were 0.46, 3.97, 0.49, and 3.12 mM, respectively, and the IC_{50} values of Cd(II) were 0.47, 1.02, 0.98, and 0.74 mM. Based on the IC_{50} values, dye decolorization in the cell-free culture broth of CBR43 was most severely inhibited by Cd(II), and the inhibitory effect of NaCl was relatively low.

Yan *et al*. [25] previously isolated *T. trogii* S031, which is tolerant against the high temperature of 80°C and highly concentrated ionic solutions. They prepared a crude laccase solution from this fungus and studied the effect of NaCl on its decolorization activity for malachite green. The decolorization efficiency for malachite green decreased less than 10% when 100 mM NaCl was added to the crude laccase solution [25]. In addition, the dye decolorization activity of a peroxidase purified from *Perenniporia subacida*, an alkali-resistant fungus, could be maintained at 60% with the addition of 100 mM NaCl [28]. The dye decolorization activity of the laccase purified from *T. pubescens* was not inhibited by 25 mM NaCl [21]. However, similar to the results of this study, the decolorization abilities of most fungi, such as *Trametes* sp. [19], *T. trogii* [18], and *Ganoderma lucidum* [29], were sensitive to inhibition by a NaCl concentration less than 50 mM.

The effects of heavy metals on the dye decolorization ability of various fungi differed according to the type of heavy metal as well as the type of fungus. Mn(II), Mg(II), and Cu(II) slightly inhibited the dye decolorization by laccase from *Trametes* sp. at the low concentration of 1 mM [18, 20], but Fe(II) inhibited the dye decolorization ability of *Trametes* sp. even below 1 mM [26]. In this study, the dye decolorization ability of *Trametes* sp. was inhibited by Zn(II) and Cd(II) at concentrations of less than 10 mM (Fig. 3), but an earlier study found that the dye decolorization ability of *T. trogii* Berk S031 was inhibited less than 10% by 100 mM Zn(II) [25]. Little inhibition was observed in the dye decolorization activity of laccase purified from *T. pubescens* with 25 mM Zn(II) [21].

Most of the previous research on dye decolorization using white-rot basidiomycetes evaluated decolorization efficiencies by reaction time and lacked kinetic analysis data. Moreover, previous research on the effects of inhibitors such as NaCl and heavy metals on dye decolorization focused on dye decolorization efficiency at various concentrations of inhibitors. Generally, the toxicity of inhibitors has been evaluated based on lethal concentration or inhibition concentration. Therefore, the kinetic information and IC_{50} values for the inhibitors of dye decolorization obtained in this study can be used to quantitatively evaluate the inhibitor effects on dye decolorization using *T. versicolor* and to design future dye wastewater treatment processes.

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References

1. Singh H. 2006. *Mycoremediation: Fungal Bioremediation,* pp. 420-472. John Wiley & Sons, New Jersey.
2. Singh RL, Singh PK, Singh RP. 2015. Enzymatic decolorization and degradation of azo dyes – a review. *Int. Biodeterior.*

Biodegradation **104:** 21-31.
3. Wesenberg D, Kyriakides I, Agathos SN. 2003, White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnol. Adv.* **22:** 161-187.
4. Cho KS, Ryu HW. 2015. Biodecolorization and biodegradation of dye by fungi: a review. *Kor. Soc. Biotechnol. Bioeng. J.* **30:** 203-222.
5. Heinfling A, Bergbauer M, Szewzyk U. 1997. Biodegradation of azo and phthalocyanine dyes by *Trametes versicolor* and *Bjerkandera adusta*. *Appl. Microbiol. Biotechnol.* **48:** 261-266.
6. Young L, Yu J. 1997. Laccase-catalyzed decolorization of synthetic dyes. *Water Res.* **31:** 1187-1193.
7. Yang XQ, Zhao XX, Liu CY, Zheng Y, Qian SJ. 2009. Decolorization of azo, triphenylmethane and anthraquinone dyes by a newly isolated *Trametes* sp. SQ01 and its laccase. *Proc. Biochem.* **44:** 1185-1189.
8. Rodríguez Couto SR, Toca Herrera JL. 2006. Industrial and biotechnological applications of laccases: a review. *Biotechnol. Adv.* **24:** 500-513.
9. Winquist E, Moilanen U, Mettälä A, Leisola M, Hatakka A. 2008. Production of lignin modifying enzymes on industrial waste material by solid-state cultivation of fungi. *Biochem. Eng. J.* **42:** 128-132.
10. Eaton D, Chang HM, Kirk TK. 1980. Fungal decolorization of Kraft bleach plant effluent. *Tappi J.* **63:** 103-109.
11. Fujian X, Hongzhang C, Zuohu L. 2001. Solid-state production of lignin peroxidase (LiP) and manganese peroxidase (MnP) by *Phanerochaete chrysosporium* using steam-exploded straw as substrate. *Bioresour. Technol.* **80:** 149-151.
12. Hakala TK, Lundell T, Galkin S, Maijala P, Kalkkinen N, Hatakka A. 2005. Manganese peroxidases, laccases and oxalic acid from the selective white-rot fungus *Physisporinus rivulosus* grown on spruce wood chips. *Enzyme Microb. Technol.* **36:** 461-468.
13. Kachlishvili E, Penninckx M, Tsiklauri N, Elisashvili V. 2006. Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation. *World J. Microbiol. Biotechnol.* **22:** 391-397.
14. Levin L, Herrmann C, Papinutti VL. 2008. Optimization of lignocellulolytic enzyme production by the white-rot fungus *Trametes trogii* in solid-state fermentation using response surface methodology. *Biochem. Eng. J.* **39:** 207-214.
15. Li H, Zhang R, Tang L, Zhang J, Mao Z. 2015. Manganese peroxidase production from cassava residue by *Phanerochaete chrysosporium* in solid state fermentation and its decolorization of indigo carmine. *Chinese J. Chem. Eng.* **23:** 227-233.
16. Liang YS, Yuan XZ, Zeng GM, Hu CL, Zhong H, Huang DL, *et al*. 2010. Biodelignification of rice straw by *Phanerochaete chrysosporium* in the presence of dirhamnolipid. *Biodegradation* **21:** 615-624.
17. Pinto PA, Dias AA, Fraga I, Marques G, Rodrigues MAM, Colaço J, *et al*. 2012. Influence of ligninolytic enzymes on straw saccharification during fungal pretreatment. *Bioresour. Technol.* **111:** 261-267.
18. Grassi E, Scodeller P, Filiel N, Carballo R, Levin L. 2011. Potential of *Trametes trogii* culture fluids and its purified laccase for the decolorization of different types of recalcitrant dyes without the addition of redox mediators. *Int. Biodeterior. Biodegradation* **65:** 635-643.
19. Manel MK, Hela ZM, Lassaad B, Steve W, Tahar M. 2009. Malachite green decolourization and detoxification by the laccase from a newly isolated strain of *Trametes* sp. *Int. Biodeterior. Biodegradation* **63:** 600-606.
20. Mechichi T, Mhiri N, Sayadi S. 2006. Remazol brilliant blue R decolourization by the laccase from *Trametes trogii*. *Chemosphere* **64:** 998-1005.
21. Si J, Peng F, Cui B. 2013. Purification, biochemical characterization and dye decolorization capacity of an alkali-resistant and metal-tolerant laccase from *Trametes pubescens*. *Bioresour. Technol.* **128:** 49-57.
22. Cho KS, Lee JI, Hong S, Jung HK, Kim KW, Min DH, Ryu HW. 2016. Novel dye-decolorizing fungus, *Bjerkandera adusta* NIBRKDFPFGC000002676, and method for treating dye wastewaters using the same. Korean Patent 10-2016-0048219.
23. Baccar R, Blanquez P, Bouzid J, Feki M, Attiya H, Sarra M. 2011. Decolorization of a tannery dye: from fungal screening to bioreactor application. *J. Biochem. Eng.* **56:** 184-189.
24. Bibi I, Bhatti HN. 2012. Biodecolorization of reactive black 5 by laccase mediator system. *Afr. J. Biotechnol.* **11:** 7464-7471.
25. Yan J, Niu J, Chen D, Chen Y, Irbis C. 2014. Screening of *Trametes* strains for efficient decolorization of malachite green at high temperatures and ionic concentrations. *Int. Biodeterior. Biodegradation* **87:** 109-115.
26. Wong L, Yu J. 1999. Laccase-catalyzed decolorization of synthetic dyes. *Water Res.* **33:** 3512-3520.
27. Rodríguez Couto S, Sanromán MA. 2005. Application of solid-state fermentation to ligninolytic enzyme production. *Biochem. Eng. J.* **22:** 211-219.
28. Si J, Cui BK. 2013. A new fungal peroxidase with alkaline-tolerant, chloride-enhancing activity and dye decolorization capacity. *J. Mol. Catal. B Enzym.* **89:** 6-14.
29. Zilly A, Coelho-Moreira JD, Bracht A, de Souza CGM, Carvajal AE, Koehnlein EA, Peralta RM. 2011. Influence of NaCl and Na_2SO_4 on the kinetics and dye decolorization ability of crude laccase from *Ganoderma lucidum*. *Int. Biodeterior. Biodegradation* **65:** 340-344.