NOTES

Screening and Investigation of Dye Decolorization Activities of Basidiomycetes

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Abbreviation: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

The decolorization of industrial dyes was investigated using extracellular enzymes produced by 21 basidiomycetes, mainly edible mushrooms. Among the 27 dyes used in this study, nine were decolorized by over 40%. Most fungi decolorized Acid Orange 20, but they showed different specificities in the case of the other dyes. Determination of activity staining by native polyacrylamide gel electrophoresis revealed that all the decolorization activities corresponded to ABTS oxidation activities.

Industrial dyes are released into the environment as wastewater by industries. The colored water evokes an unpleasant feeling even if it is only slightly visible. Moreover, some of them are stable against light, temperature and biodegradation, and have accumulated in the environment as recalcitrant compounds. Bioremediation may be the most effective method of treating such contaminants. However, dyes within wastewater are poorly decolorized by aerobic biotreatments (1, 2), and thus, useful microbes or enzymes that are readily available for conventional processing should be developed.

White-rot fungi are a class of microorganisms that produce efficient enzymes capable of decomposing dyes under aerobic conditions. They produce various oxidoreductases that degrade lignin and related aromatic compounds. They are also expected to be used for the degradation of many pollutants such as dioxins (3), PCB (4), and other recalcitrant or toxic substances (5, 6). Particularly, the decolorization of azo dyes has been mainly studied (7, 8). In the study of phenol oxidoreductases, industrial dyes are expected to be an available substrate because most of them are aromatic compounds with various substitution groups, and many types with a structural resemblance are available on the market. Moreover, the ability of enzyme to degrade such dyes can be easily determined by measuring the dye decolorization. However, there are few studies on the possibility of dye decomposition except for the monoazo group. In addition, microorganisms involved in degradation have been limited to certain fungi. In this study, the decolorization ability of 27 types of dye was investigated using the extracellular enzymes produced by 21 basidiomycetes. Furthermore, the substrate specificity and the relationship of the enzymes to the ligninolytic enzymes were determined.

Table 1 shows the 27 dyes used in this study and their abbreviations. The basidiomycetes shown in Table 2 were inoculated into 15 g of wheat bran with 64% moisture in a 100 ml Erlenmeyer flask. The culture was carried out at 20°C for 30 d. Extracellular enzymes were prepared at 4°C by stirring the medium at 120 rpm with 50 ml of 20 mM ammonium acetate buffer (pH 5.0) in the flask. After 10 min, the suspension was centrifuged at 15,000 rpm at 4°C for 30 min and the supernatant was used for the experiment. **←Table 1 and 2**

The decolorization activities of the enzymes produced by the 21 basidiomycetes were investigated using 27 types of dye at pHs 3.0, 5.0 and 7.0. These were determined by measuring the absorbance of the dye at λ_{max} (λ_{max} at pH 5.0 is indicated in Table 1). After the incubation of the reaction mixture for 24 h at 30°C in the dark, the percentage decrease in absorbance was determined using a microplate reader (Sunrise Rainbow Thermo; Tecan, Lausanne, Switzerland). Among the 27 dyes, only nine shown in Table 3 were decolorized by more than 40% by any fungus. The decolorization might have been caused by enzyme action because the decolorization was not shown using a heat-denatured extract. Among all the dyes, AO20 was strongly decolorized by most fungi at approximately pH 3.0 to 5.0. However, various specificities were observed in the case of the other dyes. *Lentinus edodes* showed high activity against all dyes listed in Table 3. *Agaricus bisporus*, *Armillariella mellea*, *Lampteromyces japonicus*, *Panellus serotinus* and *Pleurotus ostreatus* decolorized both phthalocyanine and triphenylmethane dyes by more than 70%. *Pleurotus salmoneostramineus* was most effective for the two phthalocyanine dyes, and the decolorization reached more than 90% (DB86 and PB). The optimum

pHs for the decolorization of these dyes were also around 3.0 to 5.0.

To investigate the relationship between the decolorization and the ligninolytic enzyme activities, the six fungi that had different decolorization activities were used for the following experiment. Lignin peroxidase (LiP) and manganese peroxidase (MnP) activities were assayed according to a previous report (9). The ABTS oxidation activity (10) was assayed as an index of laccase (Lcc) and other oxidoreductases. All fungi showed ABTS oxidation activity but not other ligninolytic enzyme activities. The ABTS activities were 53 (*A. bisporus*), 5.0 (*L. japonicus*), 19 (*L. edodes*), 0.83 (*P. serotinus*), 6.2 (*P. ostreatus*) and 1.9 (*P. salmoneostramineus*) U/ml. The low MnP activity (0.26 U/ml) was found only in the case of *P. ostreatus.* Particularly, *A. bisporus* and *L. edodes* showed high activity against ABTS. However, these did not correlate with the dye decolorization activity at all. These facts suggest that the decolorization might be caused by Lcc or other types of oxidoreductase with different substrate specificities. Among these fungi, the characteristics or the gene sequences of some Lcc produced by *A. bisporus* (11, 12), *L. edodes* (13) and *P. ostreatus* (14) have been reported. Particularly, Lcc 1 from *L. edodes* was reported to be effective in some dyes without the need for mediators (15). Some Lcc isozymes also show different specificities against various phenolic compounds (16). **←Fig. 1**

The determination of activity staining by native polyacrylamide gel electrophoresis was performed using the four dyes that decolorized well (Fig.1). These dyes, namely AO20, DB1, DB86 and BG1, are monoazo, diazo, phthalocyanine and triphenylmethane dyes, respectively. After electrophoresis under alkaline native conditions, the gel was incubated for 15 min in 20 mM sodium acetate buffer (pH 5.0) containing 2 mM ABTS, 25 mM AO20, 0.6 mM DB1, 25 mM DB86 or 10 mM BG1. In the case of the dyes, the gel was then transferred to the same buffer lacking the dye and incubated until the band appeared. When ABTS was used as a substrate, one or more activity bands were observed in most fungi. Some of the activity bands showed diffused or heterogeneous bands with different mobilities. These might be some isoforms with different glycosylation or modifications such as by proteolysis. When dye was used as a substrate, decolorized and discolored bands were observed, the latter also being decolorized after further incubation. All activity bands corresponded completely to that of ABTS oxidation. For all the fungi used, the activity bands were divided into two groups according to their specificity. The first included bands B, E, F, G, J, K and L, which showed a wide specificity against most dyes used in Fig. 1. Exceptionally, bands J and L had no ability to decolorize BG1. The second group, including bands A, C, D, H, I and M, was almost specific for some dyes. Among these, all bands except for C showed the ability to decolorize AO20 alone. A pair of these groups was present in all the fungi except for *L. edodes*, which abundantly showed a single decolorization activity. It seemed that the F band showed higher activity against ABTS than the other dyes. In contrast, bands J and L decolorized the dyes well despite the low ABTS activity. In the assay by absorbance measurement, all the fungi decolorized all the dyes (data not shown). The decolorization developed proportionally with incubation time.

The chemical structure of the dyes affected the decolorization. AO7 was negligibly decolorized and the decolorization percentage was less than 20% for all fungi. Its structure is very similar to that of AO20, but the hydroxyl group in the naphthalene ring binds to a different site (*o*-position). A similar result was also

observed in the case of diazo dyes. DB1 and DB15 contain the same methoxyl groups but the positions of the sulfo groups are different. DB6 also has the same structure as DB15 except for the absence of methoxyl groups. Among these dyes, only DB1 was decolorized well. In the case of triphenylmethane dyes, the length of the alkyl side chain might affect the decolorization. BG1, which contains diethylamino groups, showed more decolorization than BB1 and BV3 which contain dimethylamino groups. These structures might be similar to that of phenylpropanoid, a major component of lignin. To investigate the structure essential for decolorization, further studies using more types of dye would be necessary. The information that we obtained might be applicable to the design of various biodegradable compounds.

In this experiment, some effective enzymes for decolorization were selected. These are expected to be used not only for dye decolorization but also for the removal of other contaminants. The addition of a mediator also extends the reactivity widely, and allows for the decomposition of various substances (17).

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Figure legend

FIG. 1. Activity staining against ABTS oxidation and dye decolorization. Native PAGE was performed for 3 μg of the protein using 7.5% polyacrylamide gel. The gels were incubated with ABTS (lane 2), AO20 (lane 3), DB1 (lane 4), DB86 (lane 5) and BG1 (lane 6) at 30°C. Proteins were stained with CBB RS-250 (lane 1). The decolorized bands are indicated by the bands A-M.

TABLE 1. List of dyes used in this study

Dye	Color Index no.	λ_{max}* (nm)	Dye	Color Index no.	λ_{max}* (nm)
Monoazo dyes			Phthalocyanine dyes		
Acid Orange 20 (AO20)	14600	476	Direct Blue 86 (DB86)	74180	623
Acid Orange 7 (AO7)	15510	485	Phthalocyanine Blue (PB)	74220	624
Pigment Red 57 (PR57)	15850	502			
Acid Red 26 (AR26)	16150	502	Triphenylmethane dye		
Acid Violet 3 (AV3)	16580	552	Basic Blue 1 (BB1)	42025	630
Mordant Blue 13 (MB13)	16680	525	Basic Green 1 (BG1)	42040	626
Acid Red 1 (AR1)	18050	510	Basic Violet 2 (BV2)	42520	545
			Basic Violet 3 (BV3)	42555	587
Diazo dyes			Acid Blue 90 (AB90)	42655	583
Direct Blue 2 (DB2)	22590	568	Mordant Blue 1 (MB1)	43830	438
Direct Blue 6 (DB6)	22610	575	Mordant Blue 25 (MB25)	43825	434
Direct Blue 3 (DB3)	23705	534	Acid Blue 9 (AB9)	42090	625
Direct Blue 14 (DB14)	23850	594	Acid Green 9 (AG9)	42100	633
Direct Blue 15 (DB15)	24400	596			
Direct Blue 1 (DB1)	24410	619	Triazine dyes		
			Basic Dye (BD)	52010	645
Acridine dye			Basic Blue 9 (BB9)	52015	663
Basic Orange 14 (BO14)	46005	471			

*λ_{max} was determined at pH 5.0. The abbreviations of the dyes are shown in parentheses.

TABLE 2. List of basidiomycetes used in this study

No.	Name	No.	Name
1	*Agaricus bisporus*	12	*Mycoleptodonoides aitchisonii*
2	*Armillariella mellea*	13	*Naematoloma sublateritium*
3	*Coriolus versicolor*	14	*Panellus serotinus*
4	*Flammulina velutipes* (cultivation type)	15	*Pholiota aurivella*
5	*Flammulina velutipes* (wild type)	16	*Pholiota malicola*
6	*Grifola frondosa*	17	*Pholiota nameko*
7	*Ischnoderma resinosum*	18	*Phyllotopsis nidulans*
8	*Lampteromyces japonicus*	19	*Pleurotus eryngii*
9	*Lentinus edodes*	20	*Pleurotus ostreatus*
10	*Lentinus lepideus*	21	*Pleurotus salmoneostramineus*
11	*Lyophyllum ulmarium*		

TABLE 3. Dye decolorization activities of crude extracts from basidiomycetes

Fingi no.	Monoazo dyes									Diazo dyes						Phthalocyanine dyes						Triphenylmethane dyes					
	AO20			AV3			MB13			DB1			DB14			DB86			PB			BG1			AG9		
	pH 3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7	3	5	7
1	+++	+++	++	++	++	+	++	++	++	++	++	++	+	++	—	+++	+++	—	+	+++	—	—	+++	+	++	++	++
2	++	+++	+	+	++	+	+	++	+	+	++	+	+	++	—	+	+++	—	++	++	—	—	+++	+	++	++	+
3	+	++	+	+	+	+	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—	—	—	+	—	—	—
4	+	++	++	+	—	—	+	+	+	—	—	+	+	+	—	—	—	—	—	—	—	—	+++	+	+	—	+
5	++	+++	++	++	+	+	++	+	++	+	—	—	+	+	—	++	—	—	++	—	—	++	+++	+	+	++	++
6	++	++	—	—	—	—	—	+	—	+	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—
7	+	++	—	+	+	—	+	+	—	—	—	—	+	—	—	—	—	+	—	—	—	+	—	+	—	—	—
8	+++	+++	++	++	++	+	++	++	+	++	+	—	++	++	+	+++	+	—	—	—	—	—	+++	+	+	—	+
9	+++	+++	++	+++	++	++	++	++	++	+++	+++	+	+++	++	—	+++	+++	—	+++	++	—	—	+++	+++	+++	++	—
10	++	+	—	—	—	+	+	+	—	+	—	—	+	+	—	—	+	—	—	—	—	—	—	+	+	—	+
11	+	++	++	+	+	—	+	+	+	+	+	—	—	+	—	—	—	—	+	—	—	—	—	+	—	—	—
12	+++	++	++	+	+	—	++	++	—	++	+	+	+	+	—	+	+	—	+	—	—	—	+++	+	++	++	+
13	++	+++	+	+	+	—	+	++	++	+	—	+	+	+	+	+	+	—	++	—	—	+	—	+	—	—	+
14	+++	+++	—	++	++	+	++	+	+	—	+	+	+	+	+	+++	++	—	+++	—	—	—	+++	+	++	++	+
15	+	+++	++	+	++	—	+	++	+	+	+	+	+	+	—	—	+	—	+	—	—	—	++	+	+	—	—
16	++	+++	+	+	+	—	+	+	+	+	+	—	+	+	—	—	—	+	+	+	—	—	—	+	—	—	—
17	++	+++	+	+	++	+	+	++	+	+	+	+	+	+	—	+	—	—	+	—	—	—	+	+	—	—	+
18	++	+++	+	+	+	—	+	+	++	—	+	+	+	+	—	+	—	+	+	—	—	—	—	+	—	—	—
19	+	+++	++	+	++	+	+	++	++	—	++	+	—	+	+	+	+++	—	—	+++	—	—	+	+	—	—	+
20	+	+++	++	+	++	+	+	++	++	+	++	+	+	++	+	+	+++	—	—	++	—	+	+++	++	—	+	+
21	+	+++	++	+	++	+	+	++	++	+	+++	++	+	++	+	+	+++	+	+	+++	+	—	+	+	—	++	+
Ctl [a]	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	++	—	—	—

The reaction mixture (0.1 ml) consisted of the crude extract containing 0.6 μg of protein and 0.1 mM dye in 10 mM Britton-Robinson's buffer (pH 3, 5 or 7, respectively). The activities are indicated as the decolorization percentage: 0-9%, —; 10-40%, +; 41-70%, ++; 71-100%, +++. The abbreviations of the dyes follow those shown in Table 1.

[a] The heat-denatured crude extracts were incubated with the dyes.

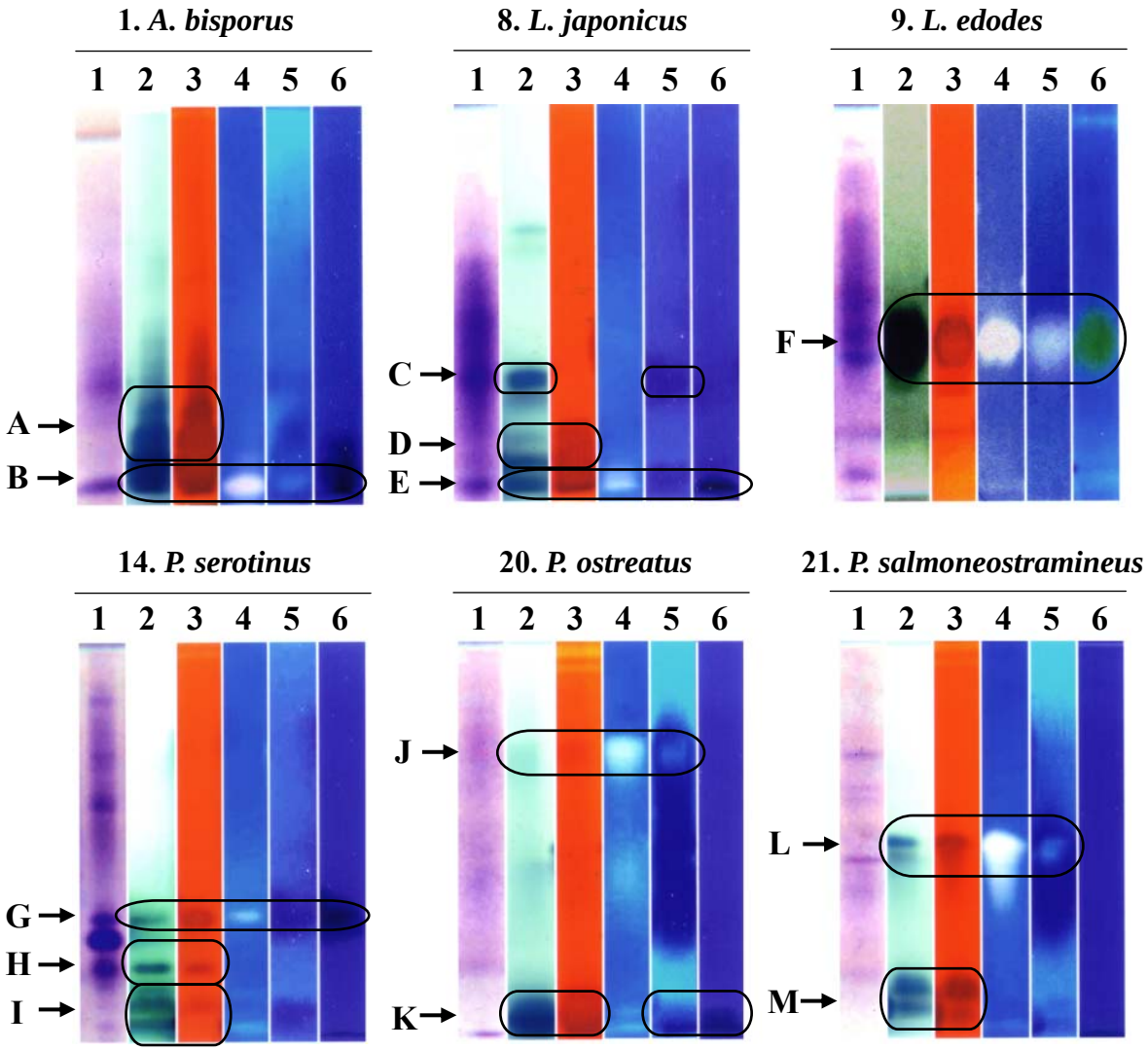

FIG 1 Nozaki *et al.*