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Decolorization of synthetic dyes by a new manganese peroxidase-producing white rot fungus

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Abstract

The decolorization of 12 different azo, diazo and anthraquinone dyes was carried out using a new isolated white rot fungus, strain L-25. A decolorization efficiency of 84.9–99.6% was achieved by cultivation in 14 days using an initial dye concentration of 40 mg L^{-1}. The strain L-25 produces manganese peroxidase (MnP) as its major ligninolytic enzyme. The adsorption of dye by cells was observed during the decolorization at the beginning of the process. However, this color disappeared when MnP was released by the strain L-25. The activity of MnP in the cultures was over 1.0 U mL^{-1} at the end of cultivation. Meanwhile, MnP produced by strain L-25 was used for the enzymatic decolorization of the dyes thus confirming the capability of the enzyme for this purpose.

Keywords: Decolorization; Dyes; Manganese peroxidase; White rot fungus

1. Introduction

Dyes have been extensively used in a broad range of industries, especially in textiles. An inevitable loss occurs during dyeing processes, in some cases up to 15% [1]. The decolorization of colored wastewaters is important not only because of the color effect, but also because many dyes are believed to be toxic, carcinogenic [2] or to be prepared from known carcinogens such as benzidine or other aromatic compounds that might be reformed as a result of microbial metabolism [3]. A number of chemical and physical methods, such as ozonation [4], Fenton reaction [5] and adsorption [6] have been used for the decolorization of wastewater. However, these technologies are usually costly for the removal of color and are not easily adapted to a wide range of dye wastewaters [7]. Conventional biological wastewater treatment systems such as municipal sewerage system are also not efficient for decolorization [8]. In recent years there has been an increasing interest in white rot fungi that have been found to have the capability to degrade various xenobiotic compounds [9,10] including dyes [11]. White rot fungi produce all or some of the extracellular ligninolytic enzymes such as lignin peroxidase, laccase and manganese peroxidase (MnP), which are responsible for the degradation of pollutants. MnP (EC 1.11.1.13) which is the most common extracellular ligninolytic enzyme produced by various basidiomycetous fungi [12], is a heme containing glycoprotein that requires hydrogen peroxide as an oxidant [13].

In this study we report on the isolation of a white rot fungus producing MnP as its ligninolytic enzyme. The organism was employed in the removal of 12 different azo, diazo and anthraquinone dyes in batch cultures. The role of MnP in the decolorization process was also investigated in in vitro enzymatic experiments.

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2. Experimental

2.1. Reagents

All the dyes used in this research were obtained from Sumitomo Chemical Co. Ltd, Japan. The selected dyes were those with the highest annual production by the above company. Table 1 lists the dyes used in this study and the wavelength of maximum absorbance. Potato dextrose broth was purchased from Becton, Dickson and Company, France. Polypepton, potato dextrose agar and hydrogen peroxide were the products of Nihon Seiyaku, Japan, Nissui, Japan, and Suntoku Chemical Industries, Japan, respectively. Acetic acid and $MnSO_4 \cdot 4–5 H_2O$ were purchased from Wako Pure Chemical Industries, Japan. Malonic acid disodium salt monohydrate was obtained from Nacalai Tesque, Japan.

2.2. Microorganism

The fungal strain L-25 was selected among screened microorganisms isolated from various soil and rotted wood samples collected in the prefectures of Fukui and Hokkaido, Japan. The screening was based on the ability of each isolate to produce an orange color on plates containing 3.9% potato dextrose agar and 0.01% remazol brilliant blue R. The L-25 isolate was stored at 4 °C on 3.9% potato dextrose agar slants.

Table 1
Dyes used for the decolorization experiments and the wavelength of maximum absorbance

No.	Dye	Color index No.	Type	Wavelength of maximum absorbance (nm)
1	Direct Fast Orange S	D.[a] Orange 26	Diazo	480
2	Nippon Fast Red BB	D. Red 31	Diazo	527
3	Sumilight Supra Blue	D. Blue 71	Diazo	580
4	Suminol Milling Orange SG	A.[b] Orange 56	Diazo	413
5	Suminol Fast Red B	A. Red 6	Azo	506
6	Suminol Milling Green G	A. Green 27	Anthraquinone	606
7	Suminol Fast Blue PR conc.	A. Blue 129	Anthraquinone	629
8	Sunchromine Yellow MD	M.[c] Yellow 3	Azo	360
9	Sunchromine Fast Blue MB	M. Blue 13	Azo	534
10	Sunchromine Black ET	M. Black 11	Azo	528
11	Sumifix Brilliant Orange	R.[d] Orange 16	Azo	492
12	Sumifix Black B	R. Black 5	Azo	597

[a] Direct.
[b] Acid.
[c] Acid mordant.
[d] Reactive.

2.3. Media and inoculation

Culture broth of 100 mL (24 g L^{-1} potato dextrose broth, 1 g L^{-1} Polypepton, and 0.1 mM Mn^{2+} included in the medium, pH 7.0) was prepared in 500-mL baffled Erlenmeyer flasks and autoclaved (121 °C, 15 min). Concentrated solutions of dyes (800 mg L^{-1}) were prepared separately. The culture broth was supplemented with this dye solution to a final concentration of 40 mg L^{-1} before inoculation. One mycelial piece of strain L-25 was placed in the center of a 3.9% potato dextrose agar plate and incubated at 30 °C. After 5 days of cultivation, a mycelial plug (diameter 10 mm) from this culture was used as the inoculum. The cultivation was conducted in a rotary shaker with the rotation speed of 150 rpm at 30 °C.

2.4. Biosorption studies

Experiments were conducted to study the biosorption capability of cells. The fungus strain L-25 was cultivated in potato dextrose broth (24 g L^{-1}) under the same conditions described in Section 2.3 for 7 days. The cells were harvested, washed with generous amount of distilled water and dried at 60 °C for 24 h. Then the dried biomass was ground using mortar and pestle. Biomass (10 mg) was added to 7 mL of 40 mg L^{-1} of dye solution and agitated on a rotary shaker for 24 h. Then, the biomass was removed by centrifugation and the dye concentration was measured.

2.5. Preparation of MnP

Partially purified MnP was prepared as follows: the strain L-25 was cultivated under the same conditions as used for dye removal except that no dye was included in the medium. After observing the peak for extracellular MnP activity, the enzyme was collected from the culture by centrifugation (10,000 × g for 30 min). The supernatant was then ultra-filtered through a 10,000 Da cut-off membrane, Vivaspin20 (Vivascience, Germany) by centrifugation and washed 3 times with 0.1 M acetate buffer (pH 4.5). In this procedure, the manganous ions (Mn^{2+}) that were included in the medium as well as the hydrogen peroxide released by the cells during cultivation could be removed.

2.6. Enzymatic reaction

The in vitro enzymatic reactions for dye removal were conducted at 30 °C in 3 mL of reaction mixture containing 0.7 U mL^{-1} of partially purified MnP, 10 mg L^{-1} of dye and 0.3 mM manganous ions (Mn^{2+}) in 0.1 M acetate buffer (pH 4.5). The reaction was initiated by the addition of hydrogen peroxide (1.0 mM in the reaction mixture) and the dye concentration was determined spectrophotometrically.

2.7. Measurements

Samples (2 mL) removed from the flasks were centrifuged and the supernatant was used for the measurement of glucose, MnP and dye concentration.

The concentration of glucose was measured enzymatically using a test kit (Glucose C-II Test-Wako) obtained from Wako Pure Chemical Industries, Japan.

MnP was assayed by the formation of Mn^{3+} in 50 mM sodium malonate buffer (pH 4.5), in the presence of 0.1 mM H_2O_2. Manganic ions Mn^{3+} form a complex with malonate, which absorbs at 270 nm [14]. One unit of MnP was defined as the amount of enzyme required to form 1 μmol of Mn^{3+} in 1 min at 25 °C.

Dye concentrations were estimated from standard curves for each dye, at the wavelength of maximum absorbance.

The UV–visible spectra of the dyes in the culture medium were measured between 200 and 800 nm before and after the cultivation.

A Hitachi U-2001 spectrophotometer, Japan was used for the above colorimetric measurements.

3. Results and discussion

3.1. Decolorization by the culture of strain L-25

The decolorization of each dye samples during the cultivation of the isolated strain L-25 was investigated. The concentrations of glucose, dye and MnP were measured during a 14 day culture period. In all 12 cases the dye concentration was reduced to below 5 mg L^{-1}. Glucose was consumed as the carbon source and its concentration decreased from 23 g L^{-1} to 7.42–9.33 g L^{-1}. MnP activity was first detected at the 4th or 5th day of cultivation and its activity increased thereafter, producing over 1.0 U mL^{-1} by the end of the cultivation. No lignin peroxidase or laccase activity was detected during the cultivation. A typical time course for decolorization by strain L-25 for A. Red 6 is shown in Fig. 1. The same pattern was also observed for the other dyes. The results of decolorization of the 12 dyes are summarized in Table 2. The absorbance of the dye-containing medium at the beginning (A_0) and at the end (A_{14}) of cultivation and the medium (A_m) was obtained. The dye removal efficiency was defined as $(A_0-A_{14})/(A_0-A_m) \times 100$. Except for D. Orange 26, which had a dye removal efficiency of 84.9%, over 98% decolorization efficiencies were observed for the remaining dyes.

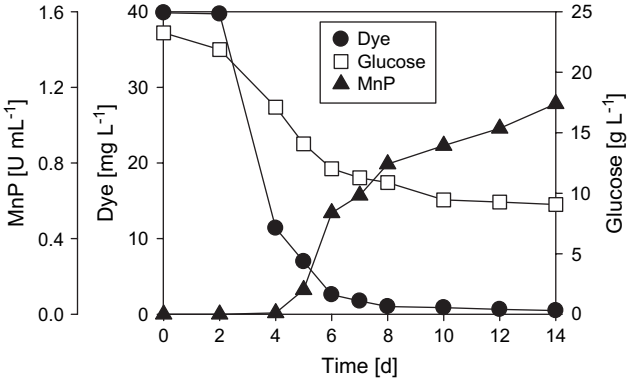

Fig. 1. Time course for decolorization by strain L-25 for A. Red 6.

In another series of cultivations, the UV–visible spectra of the dyes and culture media were obtained in the wavelength range between 200 and 800 nm. In all 12 different experiments the spectrum of the culture after 14 days of cultivation became very similar to that of a dye-free medium before cultivation. This observation confirms the ability of strain L-25 to decolorize dyes. Fig. 2 shows a typical spectrum for A. Red 6. Table 3 shows data on the absorbance of each dye, dye-free medium, medium including dye and the medium after 14 days of cultivation of strain L-25. All data are presented at the wavelength corresponding to maximum absorbance of each dye. There was a little difference in the dye removal efficiency in this case compared to that in Table 2, especially in the cases of D. Orange 26 (99.8%) and M. Yellow 3 (81.7%).

Table 2
Dye removal efficiency, glucose concentration and MnP activity in a batch culture of isolated strain L-25 using an initial dye concentration of 40 mg L^{-1} (data represent the values at the 14th day of cultivation)

No.	Dye	Glucose concentration (g L^{-1})	MnP activity (U mL^{-1})	Dye removal efficiency (%)
1	D. Orange 26	9.33	1.02	84.9
2	D. Red 31	8.92	1.11	99.5
3	D. Blue 71	9.12	1.03	98.7
4	A. Orange 56	8.76	1.11	98.0
5	A. Red 6	9.07	1.11	98.8
6	A. Green 27	8.93	1.13	98.9
7	A. Blue 129	8.29	1.20	99.1
8	M. Yellow 3	9.39	1.10	99.5
9	M. Blue 13	7.99	1.30	99.4
10	M. Black 11	7.42	1.20	98.0
11	R. Orange 16	8.01	1.27	99.6
12	R. Black 5	8.54	1.14	99.3

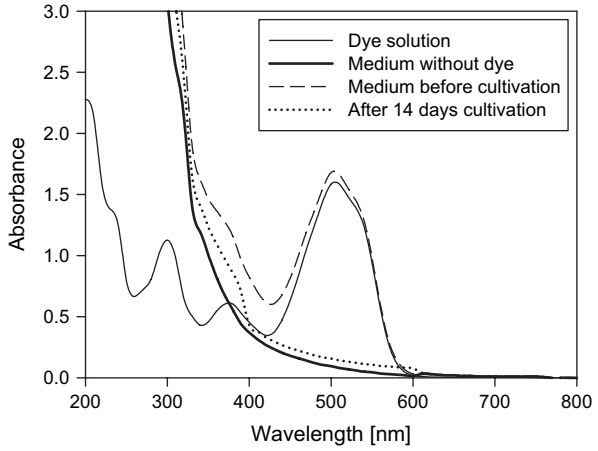

Fig. 2. UV–visible spectra of a dye solution (40 mg L^{-1}), dye-free medium and dye-containing medium before and after 14 days of cultivation, using the isolated strain L-25 against A. Red 6.

During the cultivation of strain L-25 the extent of decolorization was initially at a low level. However, about 70% of the dye was removed by the 4th day of cultivation, at which time the cells began to grow vigorously. This is consistent with the biosorption of dye occurring first, followed by the degradation of the dye. In the experiments conducted to investigate the biosorption of dyes to biomass, except for R. Orange 16, biosorption rates from 3.5% (for R. black 5) to 71.2% (for A. Blue 129) were observed for the remaining dyes.

In recent years, a number of studies have been carried out using different white rot fungi in attempts to decolorize a variety of dyes. Selected fungal isolates showed between a 56 and 100% color removal efficiency within 14 days [15]. Nine screened white rot fungal strains were used for the decolorization of brilliant green, cresol red, crystal violet, congo red and orange II, with a color removal efficiency of 20–100% in 5 days of cultivation [16]. *Phanerochaete sordida* was used for the decolorization of R. Red 120 and a decolorization efficiency of 90.6% was observed after a 7-day culture [17]. However, little information is available concerning the adsorption of dyes by cultivated cells.

Table 3
Absorbance of dye-containing media after 14 days of cultivation of strain L-25 in comparison to dye-containing and dye-free media (only the values at the wavelength of maximum absorbance of each dye are presented)

No.	Dye	Absorbance of dye solution	Absorbance of medium		
			Without dye	Before cultivation	After 14 d cultivation
1	D. Orange 26	1.035	0.120	1.155	0.122
2	D. Red 31	1.661	0.063	1.723	0.125
3	D. Blue 71	1.372	0.025	1.397	0.087
4	A. Orange 56	1.268	0.302	1.368	0.364
5	A. Red 6	1.558	0.086	1.643	0.148
6	A. Green 27	0.507	0.024	0.531	0.056
7	A. Blue 129	0.907	0.026	0.864	0.020
8	M. Yellow 3	1.473	0.825	1.573	0.962
9	M. Blue 13	1.443	0.056	1.499	0.118
10	M. Black 11	0.584	0.062	0.646	0.124
11	R. Orange 16	1.295	0.103	1.398	0.165
12	R. Black 5	1.489	0.017	1.506	0.079

During the cultivation of the strain L-25 it was found that the cells began to adsorb the color of the applied dye. As the cultivation proceeded and the extracellular MnP enzyme was released, the color disappeared from the cells. The decolorization of the liquid culture was complete at about 7 days after inoculation. However, the color of the dyes adsorbed to the cells disappeared after 10 days. Thus, a biosorption mechanism appears to be involved in the decolorization by strain L-25. Dead cells are generally thought to be dye adsorbents in decolorization processes [18]. However, dead cells are unable to degrade dyes and therefore other treatments would still be necessary after biosorption. In the cultures of strain L-25, MnP catalyzes the degradation of dye adsorbed by the cells as well as dye that remains in the liquid medium. When manganous ions (Mn^{2+}) and Polypepton were excluded from the media, the decolorization efficiency was between 66.5 and 89.4% with a slower decolorization rate (data not shown). In addition, contrary to the media containing manganous ions (Mn^{2+}) and Polypepton, at the end of the cultivation period (day 14), a mixture of both colored and non-colored cells remained in the media. This observation indicates that a portion of the dyes was removed by a biosorption mechanism under these conditions. In such cultures the maximum activity of MnP was only 0.04 U mL^{-1}. Apparently, a lack of sufficient MnP resulted in the dye being retained in the media and cells. To the contrary, the culture medium containing Polypepton (as nitrogen source) and manganous ions (Mn^{2+}), supplied sufficient nitrogen for both the growth of strain L-25 and the production of MnP, and as a result, the decolorization proceeded. Meanwhile, the presence of manganous ions (Mn^{2+}) in the medium has the stimulating effect on the production of MnP [19].

These results suggest that MnP as the main lignin degrading enzyme of strain L-25, plays a major role in the decolorization ability of strain L-25.

3.2. Enzymatic reactions

Some in vitro enzymatic experiments with MnP were performed to examine the role of MnP in dye decolorization. The dyes were reacted with partially purified MnP, obtained from the culture of strain L-25. Decolorization was observed only in the presence of MnP, manganous ions (Mn^{2+}) and hydrogen peroxide. No decolorization was observed when any of these components were not present in the reaction mixture.

Hydrogen peroxide itself had no decolorization effect at the applied conditions during the 3 min reaction time.

The decolorization of each dye was conducted in 3 mL of reaction mixture at 30 °C in 3 min. The enzymatic decolorization of D. Orange 26, A. Red 6, A. Blue 129 and M. Black 11 is shown in Fig. 3, as an example. The enzymatic decolorization efficiency for dye removal after 3 min is summarized in Table 4. The percentage of dye removal was between 16.9% for M. Yellow 3 and 98.0% for D. Blue 71. These results confirm the role of MnP in decolorization by the culture of strain L-25. The enzymatic reaction was nearly complete after 3 min, which is an extremely short reaction time compared to other reported findings. The removal of Orange II (100 mg L^{-1}) by MnP has been reported in a continuous reactor (250 mL) with a retention time of 60 min [20]. The enzymatic removal of remazol brilliant blue R and other dyes (50 mg L^{-1}, 5 mL) with MnP has been accomplished in 6 h [21]. MnP has been used for the removal of R. Black 5, R. Violet 5 and R. Blue 38 (0.025 mM, 1.0 mL) in a 20 min reaction period [22].

The proposed mechanism for the functionality of MnP involves the oxidation of manganous ions (Mn^{2+}) to Mn^{3+}, which is then chelated with organic acids. The chelated Mn^{3+} diffuses freely from the active site of the enzyme and can oxidize secondary substrates [23]. Although the efficiency of enzymatic decolorization using MnP was low for some dyes as shown in Table 4, it can be enhanced by optimizing the concentrations of hydrogen peroxide and manganous ions as well as the amount of enzyme, the pH and the reaction temperature for each dye [24]. Enzymatic decolorization might be also improved in the presence of surfactants, such as Tween 80 [25]. A number of chelators such as malonate, oxalate and L-tartrate also enhance the activity of MnP [26].

Dye-containing effluents like other wastewaters, are generated in large amounts with high flow rates.

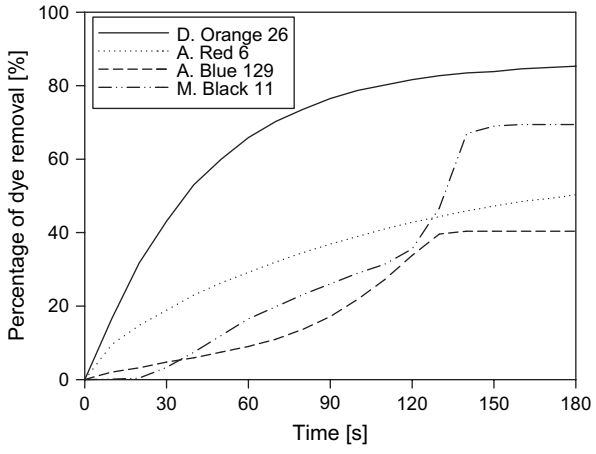

Fig. 3. In vitro decolorization of D. Orange 26, A. Red 6, A. Blue 129 and M. Black 11 by MnP.

Table 4
Percentage of dye removal, after 3 min of an in vitro enzymatic reaction using MnP (initial dye concentration was 10 mg L^{-1})

No.	Dye	Percentage of removed dye (%)
1	D. Orange 26	85.3
2	D. Red 31	95.5
3	D. Blue 71	98.0
4	A. Orange 56	46.1
5	A. Red 6	50.3
6	A. Green 27	46.3
7	A. Blue 129	40.4
8	M. Yellow 3	16.9
9	M. Blue 13	94.1
10	M. Black 11	69.4
11	R. Orange 16	37.2
12	R. Black 5	94.0

Therefore considering the 10 day culture time of the isolated strain L-25, the application of this white rot fungi for continuous decolorization would require a system with a large working volume. This problem could be overcome by the use of an enzymatic treatment system. If an appropriate method for the mass production of MnP could be developed, the enzymatic decolorization of the dye-containing wastewaters is achievable. Although, for the large scale production of MnP using white rot fungi, several problems must be overcome, such as the adverse effect of agitation on MnP activity [27], this issue has been successfully addressed in some cases involving, *Nematoloma* sp. and *Clitocybula* sp. [28]. The isolation of new species that produce MnP with a high efficiency, and engineering new species for enhanced MnP production are other challenging approaches.

4. Conclusions

A newly isolated white rot fungus, strain L-25 was used in the decolorization of 12 azo, diazo and anthraquinone dyes. The results indicated that a very high level of decolorization can be achieved using this strain. This isolate can be used in bioprocesses to remove color from industrial effluents. The role of MnP in color removal by strain L-25 was confirmed by in vitro enzymatic reactions. Moreover, the MnP produced by strain L-25 can be employed for the enzymatic treatment of colored wastewaters at the optimal reaction conditions.

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