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Screening Thermotolerant White-Rot Fungi for Decolorization of Wastewaters

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Abstract

To select a thermotolerant fungal strain for decolorization of wastewaters, ligninolytic enzyme production (lignin peroxidase, manganese peroxidase [MnP], and laccase), decolorization, and removal of total phenol and chemical oxygen demand (COD) were detected. Thirty-eight fungal strains were studied for enzyme production at 35 and 43°C on modified Kirk agar medium including 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and $MnCl_2$. Thirteen strains grew on manganese-containing agar and provided green color on ABTS-containing agar plates under culture at 43°C. Decolorization of wastewater from alcohol distillery (WAD) by these strains was compared under static culture at 43°C, and *Pycnoporus coccineus* FPF 97091303 showed the highest potential. Thereafter, immobilized mycelia were compared with free mycelia for WAD decolorization under culture conditions of 43°C and 100 rpm. The immobilized mycelia on polyurethane foam enhanced the ligninolytic enzyme production as well as total phenol and color removal. At about the same COD removal, MnP and laccase produced by immobilized mycelia were 2 and 19 times higher than by free mycelia; the simultaneous total phenol and color removal were 3.1 and 1.5 times higher than the latter. Moreover, decolorization of synthesis dye wastewater was carried out at 43°C and 100 rpm. More than 80% of 300 mg/L of reactive blue-5 was decolorized by the immobilized mycelia within 1 to 2 d for four cycles.

Index Entries: Thermotolerant fungi; *Pycnoporus coccineus*; ligninolytic enzyme; decolorization; screening.

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Introduction

Wastewater from alcohol distillery (WAD) factories that use molasses as raw material is dark brown. It consists of melanoidins from Millard reaction between carbohydrate and amino acid when heated. In addition, WAD has antioxidant properties *(1)* and resists microbial attack *(2)*. Likewise, dye wastewater from many factories also contains strong hues. There are more than 100,000 commercially available dyes, greater than with 7×10^5 t of dyestuff produced annually worldwide *(3)*, and it is estimated that about 10–15% of total dyes used in the dye processes may be in the wastewater *(4)*. Dye wastewater has high water solubility and low biodegradability *(5)*. Thus, WAD and dye wastewater are inefficiently degraded by conventional biological processes such as activated sludge treatment *(2,6–8)*.

White-rot fungi are of great interest owing to their ability to produce nonspecific extracellular ligninolytic enzymes, i.e., lignin peroxidase (LiP) (EC 1.11.1.14), manganese peroxidase (MnP) (EC 1.11.1.13), and laccase (Lac) (EC 1.10.3.2). The colored wastewaters can be decolorized by these enzymes after the cells enter secondary metabolism brought about by nitrogen or carbon starvation *(9,10)*. Generally, the optimal temperature for most fungal species is between 25 and 30°C, with lower and upper limits of about 10 and 40°C, respectively *(11)*. Several studies on enzyme and white-rot fungi for decolorization of wastewaters have been carried out under mesophilic conditions *(12–23)*. Comparatively, thermotolerant fungi have a growth temperature from below 20 to 55°C, whereas thermophilic fungi have a growth temperature minimum at or above 20°C and a maximum growth temperature at or above 50°C *(24)*.

The purposes of the present study were (1) to screen the potentially thermotolerant white-rot fungi for WAD decolorizing in solid and liquid media, (2) to compare WAD decolorizing efficiency of immobilized mycelia with that of free mycelia under thermophilic culture condition, and (3) to evaluate the decolorization of reactive dye by immobilized mycelia under sequencing batch condition and thermophilic culture condition.

Materials and Methods

Raw Materials

Concentrated WAD was supplied from an alcohol distillery plant in Osaka, Japan. Reactive blue-5 (RB-5) and other chemicals were chemical grade except polyurethane foam as supporting medium, which was purchased from general supermarkets.

Micro-Organisms and Culture Conditions

Thirty-seven fungal strains were provided by the Department of Forest and Forest Products Sciences, Faculty of Agriculture, Kyushu University, Japan, and another one (strain I-9) was isolated at Yamaguchi

University, Japan. The strains were kept on potato dextrose agar (PDA) at 4°C and subcultured every 2 mo.

Screening of Thermotolerant Fungal Strains for Ligninolytic Enzyme Production and WAD Decolorization

Two hundred and fifty milligrams per liter of 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 0.1 g/L of $MnCl_2 \cdot 4H_2O$ were added to modified Kirk medium containing 10 g/L of glucose, 0.5 g/L of ammonium tartrate, 2 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.1 g/L of $CaCl_2$, and 15 g/L of agar *(25)* as well as 20 m*M* sodium acetate for screening ligninolytic enzyme-producing fungi. The media were autoclaved at 121°C for 20 min and the initial pH was 4.5. These fungal strains were precultured on PDA plates at 35°C for 7 d until extensive mycelium growth occurred on the surface of the plates. Each plate was inoculated with one agar mycelium plug (5-mm diameter), then incubated at 35 and 43°C. The experiments were performed in triplicate.

Screening of thermotolerant fungal strains for WAD decolorization was conducted in liquid media at 43°C under static condition. Five pieces of a 5-mm-diameter plug of 7-d-old mycelia were directly inoculated in 300-mL Erlenmeyer flasks. These Erlenmeyer flasks contained 50 mL of aseptic modified Kirk medium and 3% (w/v) WAD sample (pH 4.5). The WAD medium had an initial optical intensity of 3.5 at 475 nm *(26)*.

Comparison of Decolorization Activities in WAD Between Free and Immobilized Mycelia

One-fourth of 7-d-old mycelia on a PDA plate was inoculated into 300-mL Erlenmeyer flasks containing 50 mL of aseptic modified Kirk medium and cultured for 7 d at 35°C and 100 rpm. The mycelium biomass of the selected thermotolerant strain was homogenized, washed with 0.9% NaCl solution, and used as mycelium solution. Five milliliters of the fungal mycelium solution were added to a 300-mL Erlenmeyer flask containing 50 mL of aseptic modified Kirk medium, which included 3% (w/v) WAD sample, and 0.5 g of autoclaved polyurethane foam (1 cm^3) as supporting medium for the immobilized mycelium condition. The fungal solution was incubated in a water bath shaker at 43°C and 100 rpm. Thereafter, samples were periodically taken. The experiments were performed in duplicate.

Decolorization of Reactive Dye in Wastewater by Immobilized Mycelia

RB-5 was selected for decolorizing dye wastewater by immobilized mycelia on polyurethane foam. Five milliliters of fungal mycelium solution were added to a 300-mL Erlenmeyer flask containing 50 mL of aseptic modified Kirk medium as previously described and 300 mg/L of RB-5.

Decolorization was carried out at 43°C and 100 rpm under sequencing batch condition. The experiments were performed in duplicate.

Analyses

Samples were centrifuged at 12,000*g* for 10 min at 4°C and the supernatants were collected and determined further. LiP activity was measured by the rate of oxidation of 2 m*M* veratyl alcohol in 100 m*M* sodium tartrate buffer, pH 3.0, in the presence of 0.4 m*M* H_2O_2 (ε_{310} = 9300 M^{-1} cm^{-1}) *(27)*. MnP activity was determined by the rate of oxidation of 1 m*M* 2,6-dimethoxyphenol in 100 m*M* sodium acetate buffer, pH 4.5, in the presence of 0.1 m*M* H_2O_2 and 1 m*M* $MnSO_4$ (ε_{469} = 27,500 M^{-1} cm^{-1}) *(28)*. Lac activity was determined by the rate of oxidation of 0.2 m*M* ABTS in 20 m*M* sodium acetate buffer, pH 5.0 (ε_{436} = 29,300 M^{-1} cm^{-1}) *(29)*. The enzymatic reactions were carried out at 30°C and 1 U of enzyme activity was defined as the amount of enzyme oxidizing 1 µmol of substrate/min. The pH, COD, total nitrogen, total phosphorus, and color were determined according to standard methods *(30)*. Decolorization was spectrophotometrically determined by adjusting the pH of the sample to 7.6 with NaOH or H_2SO_4 and measuring the absorbance of samples at 475 nm for WAD samples *(26)* and at 600 nm for RB-5 samples *(31)* against the initial sample.

Total phenol was determined according to the Folin-Ciocalteau method using gallic acid as the standard *(32)*. One milliliter of sample was added to approx 60 mL of distilled water in a 100-mL volumetric flask. Five milliliters of Folin-Ciocalteu reagent were added and the sample was mixed. After 7 min, 15 mL of 20% sodium carbonate solution were added and distillation water was filled to adjust the volume to 100 mL. After 2 h, the absorbance of the samples was spectrophotometrically measured at 760 nm.

Results and Discussion

Selection of Thermotolerant Fungal Strains

The initial WAD sample (3% [w/v]) contained 46 g/L of COD, 165 mg/L of total nitrogen, 574 mg/L of total phosphorus, and 1240 mg/L of total phenols (as gallic acid).

Thirty-eight fungal strains were screened for their thermotolerant potentialities in producing ligninolytic enzyme on agar plates at 35°C for the mesophilic condition and 43°C for the thermophilic condition. For the thermophilic condition, the fungi were first cultured at 45°C, but the fungi grew slowly and the maximum growth of these strains was <2 cm in diameter after 7 d. As a result, the culture temperature was reduced to 43°C, and some strains were then able to grow more than 5 cm in diameter on the agar media. Therefore, 43°C was selected as the thermophilic culture condition.

At 35°C, most fungal strains provided a green color on ABTS-containing plates and grew on manganese-containing plates (Table 1). The pres-

Table 1
Agar Plate Screenings Showing Abilities of Fungal Strains to Oxidize ABTS and Manganese As Well As Mycelium Growth for 7-d Culture (n = 3)

Strain no.	Name of strain	35°C		43°C	
		ABTS green[a]	$MnCl_2$ mycelia[b]	ABTS green[a]	$MnCl_2$ mycelia[b]
1	*Aleurodiscus disciformis* IFO-6280	+	–	+	–
2	*Bjerkandera adusta* IFO-4983	–	–	–	–
3	*Cerocorticium* sp.	–	–	+	–
4	*Oligoporus caesius*	–	–	–	–
5	*Phanerochaete chrysosporium* ME 446	+++	+++	–	–
6	*Phanerochaete sordida* TMIC 34880	+++	+++	–	–
7	*Phanerochaete sordida* YK-624	+++	+++	–	–
8	*Phanerochaete* sp.A	–	–	–	–
9	*Phanerochaete subceracea* IMIC 32054	+	++	–	–
10	*Phlebia subochracea* HHB 8494	+++	+++	–	–
11	*Phlebia subserialis* HHB 9768	+++	+++	–	–
12	*Phlebia tremellosa* BMC 9152	+	–	–	–
13	*Phlebia tremellosa* BMC 9160	+	–	–	–
14	*Phlebia tremellosa* TMIC 30511	+	–	–	–
15	*Phychoporus coccineus*	+++	+++	++	+
16	*Pycnoporus coccineus* FPF 00062506	++	+++	++	+
17	*Pycnoporus coccineus* FPF 00062507	+++	+++	++	+
18	*Pycnoporus coccineus* FPF 00062508	+++	+++	++	+
19	*Pycnoporus coccineus* FPF 01062404	+++	+++	++	+
20	*Pycnoporus coccineus* FPF 01062406	++	+++	++	+
21	*Pycnoporus coccineus* FPF 97062901	+	+++	++	+
22	*Pycnoporus coccineus* FPF 97091303	++	+++	++	+
23	*Pycnoporus coccineus* FPF 97091304	+++	+++	+	+
24	*Pycnoporus coccineus* FPF 98062802	+++	+++	++	+
25	*Pycnoporus coccineus* FPF 98063001	+++	+++	++	+
26	*Pycnoporus coccineus* FPF 99070801	++	+++	+	–
27	*Schizophyllum commune*	–	–	–	–
28	*Trametes hirsuta* YK 505	+++	++	+	–
29	*Trametes versicolor* IFO 6482	+	–	–	–
30	*Xylobolus spectabilis* IP07650	–	–	–	–
31	*Xylobolus subpileatus* IFO 7076	–	–	–	–
32	GB-1027	+	–	–	–
33	I-9	+++	+++	+	+
34	KD-070	+++	+++	+	+
35	MG-60	+	–	–	–
36	MZ-227	+	–	–	–
37	MZ-340	+	+	–	–
38	OK-190	++	+++	+	–

[a]Intensity of oxidation enzyme reaction of ABTS.
[b]Extent of radial mycelium growth on $MnCl_2$-containing agar plate.

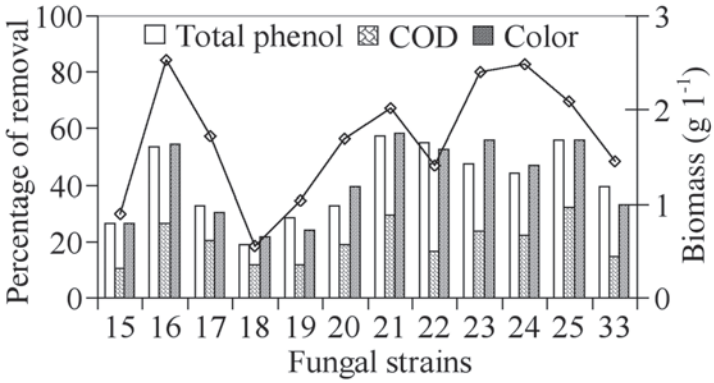

Fig. 1. Percentage of total phenol, COD, and color removal as well as biomass produced (—◇—) in WAD media by fungal strains under static culture for 9 d at 43°C ($n = 3$).

ence of extracellular radical-generating enzymes (laccases and peroxidases) was indicated by ABTS-containing plates forming dark green rings of ABTS cation radical ($ABTS^{\bullet+}$) around the fungal mycelia *(25,33)*. Manganese-containing plates were evaluated for the formation of dark brown flecks of manganese oxide (MnO_2) caused by the action of MnP *(25)*. Unlike most of the other white-rot fungi, most *Phanerochaete chrysosporium*, except *P. chrysosporium* BKM-F1767 *(34)* and *P. chrysosporium* ME 446 *(35)*, do not produce Lac; thus, the green color on the ABTS-containing plate cultured with *P. chrysosporium* ME 446 (strain no. 5) indicated the production of peroxidases and/or Lac *(25,35)*, whereas the green color on the ABTS-containing plate on which *Pycnoporus coccineus* strains were cultured indicated the production of Lac.

At 43°C, green color appeared on ABTS agar plates cultured with 18 strains and 13 strains grew on manganese-containing agar plates, whereas only strain I-9 gave the brown color of MnO_2 on $MnCl_2$ agar plates. Some strains were able to oxidize Mn^{2+} but did not illustrate MnO_2. Although Mn^{2+} was oxidized by MnP to Mn^{3+}, no MnO_2 was formed because the Mn^{3+} ions were stabilized by chelator *(36)*.

After agar-plate screening for enzyme production, 12 selected strains were further studied, but not strain no. 34, because of its low growth rate. The study was conducted in liquid medium under static culture at 43°C for 9 d. Figure 1 illustrates that fungal strains no. 16, 21, 22, and 25 removed total phenol, color, and COD by 53–57, 52–58, and 16–32%, respectively. Total phenol and color removed by strain no. 22 were almost the same as that by strain no. 21, providing the highest removal of total phenol and color, but because the biomass of fungal strain no. 22 (1.4 g/L) was lower than that of strain no. 21 (2.1 g/L), fungal strain no. 22 (*P. coccineus* FPF 97091303) was selected for further study.

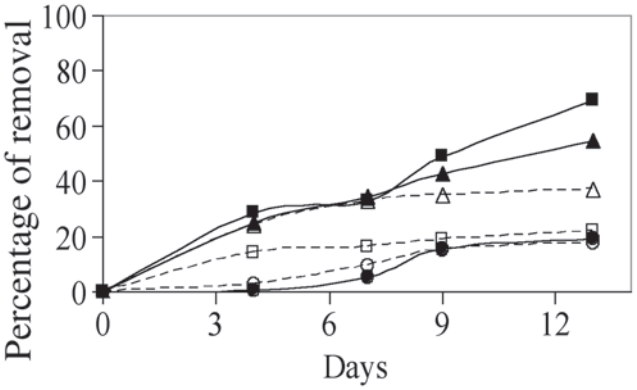

Fig. 2. Removal of total phenol (squares), color (triangles), and COD (circles) by free (dashed lines) and immobilized (solid lines) mycelia of *P. coccineus* FPF 97091303 on polyurethane foam at 43°C and 100 rpm (n = 2).

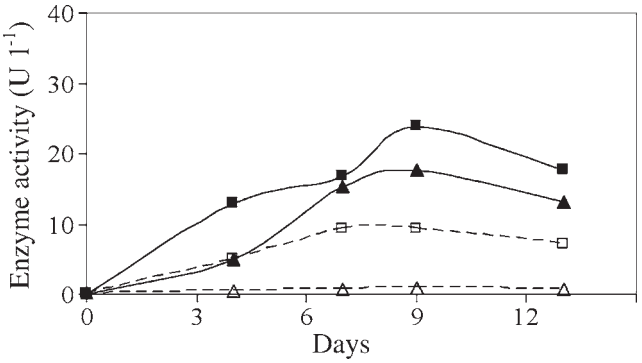

Fig. 3. MnP (squares) and Lac (triangles) activities by free (dashed lines) and immobilized (solid lines) mycelia of *P. coccineus* FPF 97091303 on polyurethane foam at 43°C and 100 rpm (n = 2).

Comparison of Decolorization Activities Between Free and Immobilized Mycelia

Based on the results of the agar plate and liquid media under static culture, *P. coccineus* FPF 97091303 was selected for further experiments because it presented the potential on decolorization at a higher temperature. Meanwhile, free and immobilized mycelia on polyurethane foam in Erlenmeyer flasks at 43°C on a shaker (100 rpm) were compared for 13 d. Figure 2 illustrates that in WAD decolorization, total phenol, color, and COD removal by immobilized mycelia were 69, 55, and 19%, respectively, higher than those by free mycelia (22, 37, and 18%, respectively). Furthermore, MnP and Lac activities produced by immobilized mycelia were 2 and 19 times higher than those obtained from free mycelia, respectively (Fig. 3). Although Jaouani et al. *(13)* reported that MnP was not detected from *P. coccineus* culture, MnP was detected in our study and the result was in

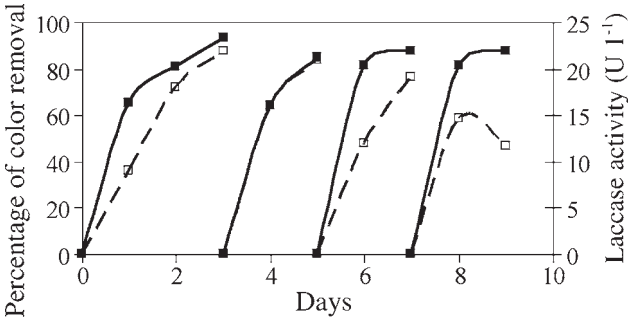

Fig. 4. Color removal (– – – –) and Lac activity (– – □ – –) by immobilized mycelia of *P. coccineus* FPF 97091303 on polyurethane foam in 300 mg/L of reactive dye wastewater at 43°C and 100 rpm (n = 2).

accordance with that of Muzariri et al. *(37)*. In addition, LiP activity could not be detected in our study, agreeing with the findings of Jaouani et al. *(13)* and Muzariri et al. *(37)*. COD removal by immobilized and free mycelia was about the same. These results suggest that the immobilized mycelia had a higher efficiency of enzyme production, decolorization, and total phenol removal than free mycelia.

Mechanically shaking culture may inhibit white-rot fungi from producing ligninolytic enzymes because of the shear stress. Immobilization of mycelium culture enhances higher activity and greater resistance to the environmental disturbances than free mycelium culture and increases the contact area between mycelia and oxygen without shear stress *(38)*. Nakamura et al. *(38)* and Fahy et al. *(39)* reported that immobilization of fungal strains on supporting media showed higher ligninolytic enzyme activities and decolorization efficiency than free mycelia.

Decolorization of Reactive Dye Wastewater by Immobilized Mycelia

Immobilized fungal mycelia of *P. coccineus* FPF 97091303 were also studied in dye decolorization, with 300 mg/L of RB-5 under sequencing batch culture at 43°C and 100 rpm. Figure 4 shows that RB-5 was decolorized >80% after 2 d in the first and second cycles and within 1 d for the third and fourth cycles. In the primary growth phase of the initial two cycles, the lag period prior to decolorization may have been owing to the need to grow to a certain biomass for enzyme production *(40)*. Lac activity was detected as more than 20 U/L in the first, second, and third cycles, but the activity was decreased to 13 U/L in the final cycle. On the other hand, MnP was not detected in the decolorization. The synthetic RB-5 wastewater contained less various nutrients than WAD wastewater, which contributed to the higher enzyme activities, owing to the nutrients respected with requirement of fungi *(41)*.

These experiments demonstrate that some *P. coccineus* strains have high potential for decolorization and biodegradation of WAD and dye

wastewater at higher temperature, although no previous study has demonstrated decolorization of these wastewaters by *P. coccineus* under thermophilic condition. Moreover, immobilization of mycelia on polyurethane foam as supporting medium could enhance their activities.

Conclusion

Screening of thermotolerant fungi is important in the selection of potential fungi for the decolorization of wastewaters under thermophilic culture condition. *P. coccineus* FPF 97091303 was 1 of 38 fungal strains that could highly degrade and decolorize WAD and dye wastewaters at 43°C. Moreover, immobilization of mycelium enhanced the fungal potential, compared with free mycelium culture. The removal of total phenol, color, and COD from WAD by the immobilized mycelia was 69, 55, and 19%, respectively, whereas that by free mycelia was about 22, 37, and 18%, respectively, after 13 d of culture under shaking condition at 43°C. Immobilized mycelia also provided MnP and Lac 2 and 19 times higher than free mycelia did under the same culture conditions. Furthermore, >80% of 300 mg/L of RB-5 was removed by the immobilized mycelia within 1 to 2 d for four cycles under sequencing batch culture even though MnP was not detected during decolorization of the dye.

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