J Wood Sci (2001) 47:63–68

NOTE

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Screening of wood-rotting fungi for kraft pulp bleaching by the Poly R decolorization test and biobleaching of hardwood kraft pulp by *Phanerochaete crassa* WD1694

Received: April 26, 1999 / Accepted: January 28, 2000

Abstract From among 419 wood-rotting fungi 10 were selected by the Poly R decolorization test, and their ability to bleach hardwood kraft pulp was assayed. Of the 10 selected, 6 fungi (i.e., *Phanerochaete crassa* WD1694 and F150; *Pleurotus pulmonarius* PSC-H, PSC-M, and PSC-T; *and Pleurotus species* A119) showed much higher bleaching ability than *Phanerochaete chrysosporium* BKMF1767 or *Trametes versicolor* WD1670, both of which are well-known high ligninolytic fungi. *P. crassa* WD1694 had the highest bleaching ability among the selected strains, and it increased the pulp brightness from 28 to 54, with a corresponding decrease in kappa number from 16 to 6 after 10 days of cultivation and alkali extraction. MnP was a predominant ligninolytic enzyme of *P. crassa* WD1694 during the biobleaching.

Key words Fungi · Screening · Poly R · Lignin · Kraft pulp · Bleaching

Introduction

The kraft pulping process has been used extensively as a commercial chemical delignification method. It requires multistage bleaching sequences to produce the final paper products because of the dark color associated with the residual lignin in the kraft pulp. Although the combination of chlorination and alkaline extraction has resulted in an efficient, highly selective bleaching method, the effluent from such bleaching stages contains many toxic chlorinated organic compounds, including dioxins, which have created one of the most serious environmental problems in the pulp and paper industry.

Studies of biobleaching seeking an alternative way to eliminate or reduce the use of chlorine-based chemicals have been reported recently. White-rot fungi are the most studied microorganisms for biobleaching because of their high, selective ability to delignify wood.[1–5] Therefore, most of the fungi studied for biobleaching were selected from high ligninolytic strains.[6–8]

We used the Poly R decolorization test for the preliminary screening of wood-rotting fungi for biobleaching kraft pulp. It is well known that many dyes are decolorized by ligninolytic organisms.[9–14] Glenn et al. reported that polymeric dyes had some advantages over model compounds of lignin, especially for screening ligninolytic fungi.[13,14]

We selected several strains using the Poly R decolorization test and examined their bleaching abilities. We also studied the ligninolytic enzymes secreted and the effect of cultivation conditions on biobleaching by *Phanerochaete crassa* WD1694, the fungus that showed the highest bleaching ability.

Materials and methods

Fungi

The fungi used were from collections of the Forestry and Forest Products Research Institute and were maintained on potato dextrose agar (PDA) plates at 4°C. Altogether, 429 fungi were cultivated on the plate media containing saccharose 5 g/l, $NH_4H_2PO_4$ 1 g/l, KH_2PO_4 360 mg/l, thiamine·HCl 10 mg/l, agar 25 g/l, Poly R-478 0.05%. After cultivation for 14 days at 26°C, 119 fungi that showed Poly R decolorization were selected.

Pulp

Hardwood kraft pulp (HWKP) (A) was prepared from beech chips in a laboratory autoclave. Cooking conditions

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This research was presented in part at the 46th Annual Meeting of the Japan Wood Research Society, Kumamoto, April 1996

were as follows: active alkali 13.5% on chips; sulfidity 25%; liquor/wood ratio 4l/kg; maximum cooking temperature 170°C; time to maximum temperature 90min; cooking time at maximum temperature 90min. Total yield was 49.4%. Kappa number was 20, and brightness was 27.0.

HWKP (B) (kappa number 12.7, brightness 34.8) and oxygen-alkali bleached kraft pulp (OKP) (kappa number 9.1) were obtained from a mill of Nippon Paper Industries.

Materials

Poly R-478 and PDA were purchased from Sigma and DIFCO, respectively. All other reagents were of standard grade.

Poly R decolorization test in the presence of pulp

Each strain of the 119 fungi selected as described above was cultivated on a PDA plate. A disk was punched from the edge of the growing mycelium and inoculated to the center of a testing plate containing 1% OKP and 0.05% Poly R-478. They were cultivated at 26°C for 7 days, and the diameters of the circles decolorized by the fungi were measured.

Fungal treatment of pulp

The liquid medium (1l) contained glucose 1%, $NH_4H_2PO_4$ 0.1%, KH_2PO_4 600mg, K_2HPO_4 400mg, $MgSO_4 \cdot 7H_2O$ 0.5g, $CaCl_2$ 5mg, yeast extract 100mg. The medium (100ml each) in 300-ml Erlenmeyer flasks was inoculated with the five agar disks obtained from the previously cultivated PDA plate inoculated with the selected fungi. After being shaken for 7 days at 30°C, the mycelium pellets were homogenized. An amount of HWKP(A) equivalent to 10g dry weight in a 1-l Erlenmeyer flask was sterilized at 121°C for 20min and inoculated with 25ml of the homogenized mixture. The pulp consistency was 25%. After stationary cultivation at 26°C for 10 days, the pulp was homogenized and washed through a flat screen. The pulp was then dehydrated and dipped into 0.1 N NaOH 200ml for an hour and washed with water until the washing solution became neutral. Control pulp was treated the same as other samples except for the inoculation. Modifications of each experiment were described respectively.

Measurement of the fungal growth at different temperatures

The diameter of mycelial growth of *P. crassa* WD1694 was measured every 3°C between 19°C and 40°C after cultivation for 3 days on PDA plates. The growth of several other strains were tested in the same way for comparison.

Time course analysis of biobleaching by WD1694

The HWKP (B) (2.5g dry weight) in a 300-ml Erlenmeyer flask was sterilized, and 8ml of homogenized culture of WD1694 was added as described above. The pulp consistency was 25%. After stationary cultivation at 26°C for each day, 50ml phosphate buffer (0.01N, pH 7.0) was added to the flask and the sample was homogenized in an ice bath. Each sample was filtered, and the pulp properties were determined. The filtrate was desalted with Sephadex G-25M (Pharmacia) and analyzed for ligninolytic enzyme activity.

Enzyme assays

Manganese peroxidase (MnP) activity was determined by monitoring the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 414nm. The reaction mixtures contained ABTS 40mg/l, 2mM $MnSO_4$, 50μM H_2O_2, and 140μl of sample in 0.1M sodium lactate buffer (pH 3.5). Laccase activity was measured in the MnP assay solution without adding $MnSO_4$ and H_2O_2. Peroxidase activity was assayed in the MnP assay solution without addition of $MnSO_4$. MnP activity and peroxidase activity were corrected by subtracting the peroxidase activity and laccase activity, respectively. Lignin peroxidase was measured by monitoring the oxidation of veratryl alcohol at 310nm in a reaction mixture containing 5mM veratryl alcohol, 50μM H_2O_2, and a 140-μl sample in 0.1M sodium lactate buffer (pH 3.5). Each assay was conducted at 30°C. One katal (kat) of enzyme is defined as the amount of enzyme that oxidizes 1mol of substrate per second.

Pulp properties

The kappa number was measured according to TAPPI standard T236m-60. Viscosity was determined by TAPPI 230 om-82. Handsheets were made by filtration, and brightness was measured by the JIS P8123 method.

Results

Among 429 wood-rotting-fungi, 119 strains showed Poly R-478 decolorization within 14 days and were selected first. These 119 fungi were assayed for their Poly R-478 decolorization abilities as described above. For this screening the cultivation time was 7 days, which was the most advantageous period for comparing and observing the fungi that gave the best screening results. The decolorized area turned pale yellow or colorless depending on the strain. Figure 1 shows the distribution of the 119 fungi after Poly R-478 decolorization.

Ten fungi that decolorized Poly R for more than a 5.0cm diameter were selected: *Phanerochaete crassa* WD1694 and F150; *Pleurotus species* A119; *Pleurotus pulmonarius* PSC-T, PSC-H, and PSC-M; *Phanerochaete chrysosporium* BKMF-1767; *Coriolus brevis* F148; *Trametes versicolor* WD1670; and *Trametes hirsuta* WD1674 (Table 1).

HWKP (A) was treated with these 10 fungi, and their bleaching effects were compared (Table 1). Of the selected

10 fungi, 6 (i.e., *P. crassa* WD1694 and F150; *Pleurotus species* A119; *P. pulmonarius* PSC-T, PSC-M, and PSC-H) increased the brightness from 27.7 to more than 42.0 and decreased the kappa number from 16.4 to less than 10.0 (Table 1). The other four fungi (i.e., *P. chrysosporium* BKMF-1767; *C. brevis* F148; *T. versicolor* WD1670; and *T. hirsuta* WD1674) showed a lower bleaching effect than the other six fungi. Brighter pulp was obtained with alkali treatment that followed fungal cultivation than without alkali treatment. The brightest pulp was obtained with *P. crassa* WD1694, which increased the brightness from 28 to 54 and decreased the kappa number from 16 to 6 after the alkali treatment that followed cultivation. *Crinipellis stipitarisa* IFO30259 and *Marasmius species* A78, the fungi that showed only slight decoloration of Poly R, did not bleach kraft pulp.

The diameters of mycelial growth of the selected fungi from 19°C to 40°C were measured (Fig. 2). *P. chrysosporium* BKMF-1767 showed the best growth among the tested fungi between 25°C and 40°C. *P. crassa* WD1694 showed better growth than the other fungi between 28°C and 37°C, except for *P. chrysosporium* BKMF-1767. The best growth of *P. crassa* WD1694 was observed at 34°C. The growth speed of the tested fungi at 25°C is reflected in the results of Poly R decolorization diameter (Table 1).

Changes in brightness of HWKP (B) treated with WD1694 at 26°C and 34°C were compared (Fig. 3). *P. crassa* WD1694 made brighter pulp during the cultivation at 34°C, but the difference in brightness was generated only during the first 2 days. Brightness increased significantly from day 1 to day 5 at 34°C and from day 1 to day 7 at 26°C.

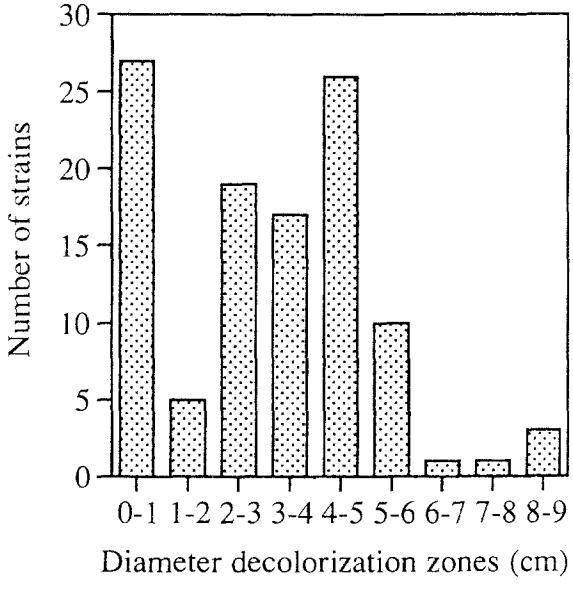

Fig. 1. Distribution of fungi on decolorization of Poly R

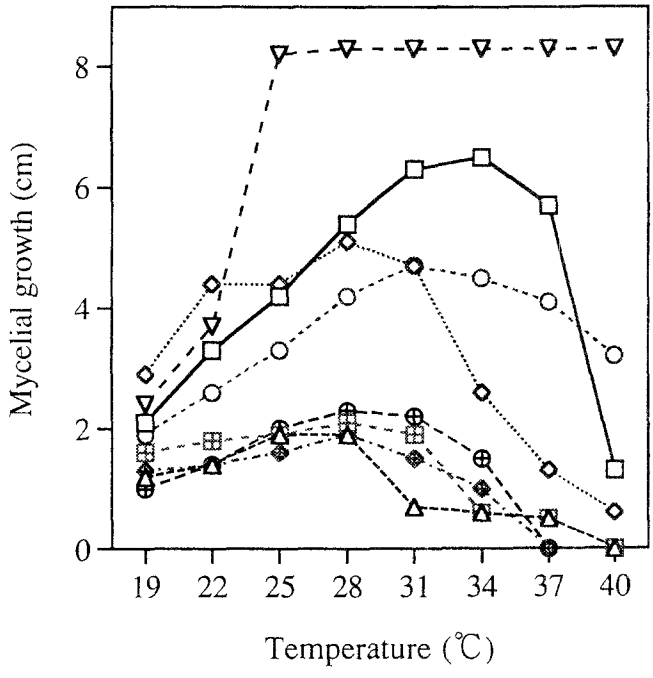

Fig. 2. Effects of temperature on mycelial growth of the selected fungi. *Open squares*, WD 1694; *open diamonds*, WD1670; *open circles*, WD1674; *triangles*, PSC-H; *crossed squares*, PSC-T; *crossed diamonds*, PSC-M; *crossed circles*, A119; *inverted triangles*, BKMF-1767

Table 1. Diameter of Poly R decoloration and kappa number and brightness of HWKP(A) treated with white rot fungi at 26°C

Strain no.	Name of strain	Diameter of Poly R decoloration (cm)	Before alkali treatment		After alkali treatment	
			Brightness (%)	Kappa no.	Brightness(%)	Kappa no.
WD1694	*Phanerochaete crassa*	8.5	48.6	7.4	54.3	6.1
F150	*Phanerochaete crassa*	5.0	41.9	9.5	45.9	7.5
A119	*Pleurotus species*	5.5	45.4	8.6	49.9	8.1
PSC-T	*Pleurotus pulmonarius*	5.0	43.1	8.7	47.8	8.3
PSC-H	*Pleurotus pulmonarius*	5.0	42.7	9.8	46.7	8.4
PSC-M	*Pleurotus pulmonarius*	5.0	43.1	10	48.1	8.3
BKMF1767	*Phanerochaete chrysosporium*	8.5	32.6	12.6	33.8	12.2
F148	*Coriolus brevis*	7.5	28.8	13.7	33.2	12.8
WD1670	*Trametes versicolor*	8.0	27.2	14.8	30.8	12.9
WD1674	*Trametes hirsuta*	6.0	28.3	14.5	31.7	13.3
IFO30259	*Crinipellis stipitaria*	2.5	24.7	16.3	28.3	14.6
A78	*Marasmius species*	0.0	25.1	16.0	28.1	15.1
Control			27.7	16.4	28.2	15.7

HWKP(A), hardwood kraft pulp (A)

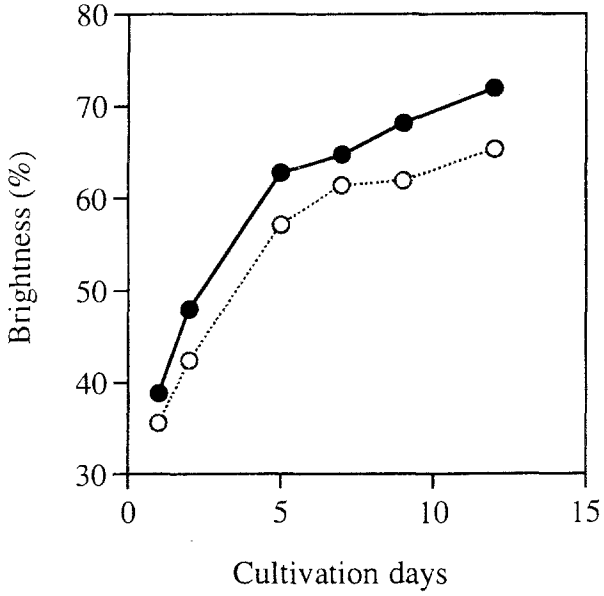

Fig. 3. Changes in brightness during the cultivation of HWKP(B) by WD1694. *Solid circles*, brightness at 34°C; *open circles*, brightness at 26°C

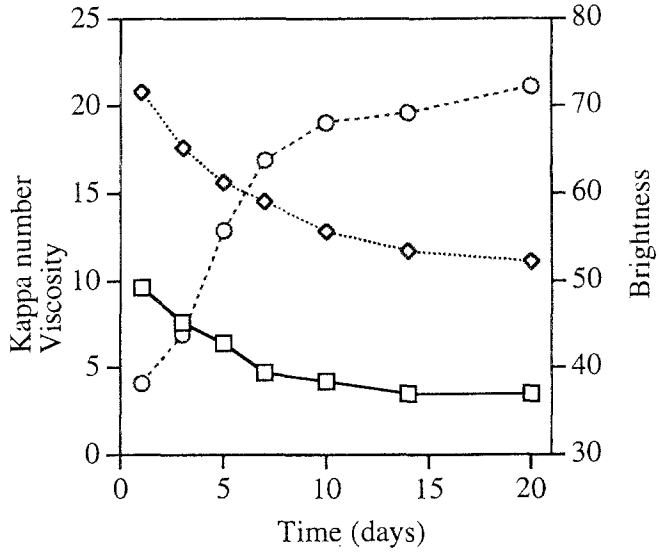

Fig. 4. Changes in the kappa number, brightness, and viscosity of HWKP(B) treated with WD1694. *Circles*, brightness; *squares*, kappa number; *diamonds*, viscosity

Table 2. Effect of 10-day treatment with WD1694 and F150 on HWKP(A)

Strain	Solid-state cultivation		Liquid-state cultivation[a]	
	Brightness (%)	Kappa no.	Brightness (%)	Kappa no.
WD1694	48.6	7.4	46.8	9.5
F150	41.9	9.5	31.7	15.8
Control	27.7	16.4	28.2	16.2

[a] Liquid-state culture was grown on a rotary shaker at 120 rpm

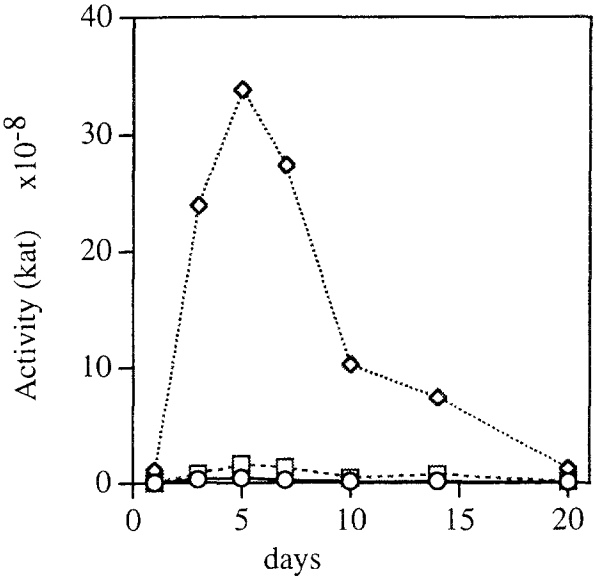

Fig. 5. Activities of ligninolytic enzymes during cultivation of HWKP(B) with WD1694. *Diamonds*, MnP; *circles*, laccase; *squares*, peroxidase

Brightness increased gradually after day 7 for both temperatures.

The effects of cultivation conditions on biobleaching by *P. crassa* WD1694 and F150 were compared. A solid-state culture containing inoculum and 25% concentration of HWKP(A) was cultivated stationary. For liquid-state cultivation, ion-exchange water was added to the solid-state culture up to 2% pulp consistency, and the culture was rotary-shaken. Both media were cultivated at 26°C for 10 days. Brighter pulp was obtained with solid-state cultivation than with liquid-state cultivation by the treatment with the two strains of *P. crassa* (Table 2). *P. crassa* WD1694 produced a pulp with a kappa number of 7.4 and brightness 48.6 during solid-state cultivation and a kappa number of 9.5 and brightness of 46.8 with liquid-state cultivation. *P. crassa* F150 showed the greatest difference in the bleaching effect treated under these conditions. The pulp treated by *P. crassa* F150 gave a kappa number of 9.5 and brightness 41.9 during solid-state cultivation and a kappa number of 15.8 and brightness 31.7 during liquid-state cultivation.

Changes in kappa number, brightness, and viscosity were monitored during cultivation of *P. crassa* WD1694 at 26°C (Fig. 4). Most of the changes in these three properties were observed during the first 10 days. Brightness increased rapidly between days 1 and 7 but only slightly after day 7. A significant decrease in kappa number was observed during the first 7 days, but there was only a small decrease between days 10 and 20. Viscosity also decreased during the first 10 days and steadily after day 10.

The ligninolytic enzymes produced were followed during the same cultivation (Fig. 5). MnP activity increased rapidly from day 1 to day 5 but dropped rapidly from day 7 to day 10. The highest MnP activity was observed on day 5. MnP activity decreased after day 10, but it was detected until day 20. Little peroxidase or laccase activity was detected. Lignin peroxidase was not detected during the cultivation.

Discussion

Many studies on the biobleaching of pulp have been reported. Most of the organisms used in these studies were selected for their high ligninolytic abilities because the color of unbleached pulp is mainly caused by lignin.[6–8]

Preliminary screening is generally used for selection from a significant number of strains to increase efficiency. Apparently the preliminary screening should be done under conditions similar to those used for the final purpose, but usually it is restricted by other factors, such as efficiency, convenience, and simplicity. The pulp plate test used by Iimori et al. gave good results because the screening condition was close to those used for pulp bleaching, but it still required further assessment to select the best strain.[15] Moreover, because pulp is not a homogeneous product, it is difficult to compare the results obtained by different pulps.

We used Poly R to select biobleaching fungi and to examine with the selected 10 fungi whether Poly R could be used for an exact determination of the degree of lignin degradation or bleaching effect. Poly R is a homogeneous reagent that is easy to obtain and use. Glenn et al. reported that the ligninolytic system was responsible for decolorization of Poly R and noted its application for lignin degradation assessment.[13,14] After this report many authors investigated the decolorization of Poly R by ligninolytic organisms or ligninolytic enzymes,[17–23] but most of these reports tested only small numbers of strains.

Recently several authors reported that MnP played an important role in the biobleaching of pulp.[3–6,16,19] Selecting fungi that produce high MnP activity may be another good way to select the fungi for biobleaching. Buckley and Dobson reported that Poly R was indicative of the presence of lignin peroxidase and MnP.[17]

Because of this background about Poly R, it is of interest to study the results of tests using Poly R as a substrate for the screening of ligninolytic fungi for biobleaching. The 10 fungi selected by the Poly R decolorization test bleached the HWKP in 10 days. Six strains from the selected 10 fungi showed much higher bleaching ability than *P. chrysosporium* BKMF-1767 or *T. versicolor* WD1670, both of which are well known highly ligninolytic fungi. These results showed that Poly R decoloration is also useful for preliminary screening of pulp-bleaching fungi. However, because the extent of Poly R decoloration did not reflect the direct bleaching ability as shown in the selected 10 fungi (Table 1), further assessment of the bleaching ability is required.

The fungus that showed the highest ability to bleach kraft pulp was *P. crassa* WD1694. Recently, several white rot fungi were screened for pulp bleaching, and their ligninolytic enzymes were investigated.[6–8,15] MnP is a major ligninolytic enzyme that has caused a bleaching effect with *Trametes versicolor*, unidentified fungus SKB-1152, and *Phanerochaete sordida* YK624.[3–4,16,19] Several properties of *P. crassa* WD1694 were similar to those of the fungi reported previously. The properties are as follows.

1. The growth rate of *P. crassa* WD1694 was better than that of other tested fungi, except for *P. chrysosporium* BKMF-1767.
2. The optimum growth temperature of *P. crassa* WD1694 was 34°C, which was higher than that for other tested fungi, except *P. chrysosporium* BKMF-1767.
3. MnP was the predominant ligninolytic enzyme during biobleaching by *P. crassa* WD1694.
4. *P. crassa* WD1694 showed much greater ability to biobleach HWKP than did *P. chrysosporium* BKMF-1767 or *T. versicolor* WD1670.

Phanerochaete crassa WD1694 increased the brightness of HWKP significantly from day 1 to day 5 at 34°C and from day 1 to day 7 at 26°C. Brightness increased slowly from day 7 to day 12 for cultivation at 26°C and 34°C. The same level of MnP was detected from days 1 to 7 for both temperatures. This could be the reason the brightness increase during cultivation at 26°C and 34°C was on a similar curve. Though the brightness at 34°C was higher than that at 26°C, the difference in brightness at the two temperatures was evident only on the first two cultivation days. Though MnP was detected at the same level for both temperatures, there remains a possibility that the MnP working system was not at the same level because MnP needs a hydrogen peroxide supply to work on the substrate. Another possibility to explain the brightness difference of the two temperatures is the growth rate difference. The growth rate at 34°C was 1.5-fold that at 26°C. The growth rate might affect the speed, amount, and distribution of the mycelia grown inside the pulp culture, and that, in turn, might cause the different distribution of MnP.

Conclusions

The effects of culture conditions on biobleaching by *P. crassa* WD1694 and F150 were compared. Although *P. crassa* WD1694 and F150 showed high bleaching ability during solid-state cultivation, a significant difference in bleaching ability appeared between the two strains when they were exposed to liquid-state culture. Because there was not a remarkable difference between the amount of the mycelium of the two strains grown in the liquid culture, the lesser bleaching effect of *P. crassa* F150 in the liquid culture was not caused by growth suppression, and it is presumed that the biobleaching system of *P. crassa* F150 might be suppressed in the liquid culture.

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